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13. ABSTRACT ***(Maximum 200 Words)***

During this career development award, I have made significant progress in determining the contribution of the α6 integrin receptors to breast cancer. We had previously established that the α6β1 receptor contributes to the growth and survival of breast carcinoma metastases. In addition, we had demonstrated that de novo expression of the integrin β4 subunit in breast carcinoma cell lines that lack this integrin subunit increases their invasive potential. During this award period, my work has resulted in an increased understanding of α6β1-dependent breast carcinoma survival and the important role of PI3K and Akt in this function. I have demonstrated that the ability of the α6β4 integrin to promote invasion is related to its activation of phosphoinositide 3-OH kinase (PI3K) and the downstream effectors Rac and PKC-ε. I identified IRS-1 and IRS-2 as intermediates in the α6β4-dependent activation of PI3K and demonstrated that Y1494 in the β4 subunit is essential for this signaling pathway. Finally, I have established an involvement of the src family in α6β4-dependent breast carcinoma invasion. Importantly, this career development award has contributed to my promotion to Assistant Professor, my recruitment to the BIDMC Pathology Department, and my obtaining an NIH-R01 Award that is focused on breast cancer.

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Introduction

Alterations in integrin expression and function during transformation are likely to have multiple consequences on tumor progression because of their adhesive and signaling properties (1,2). Our research has focused on the possible involvement of the α6 integrins, α6β1 and α6β4, receptors for the laminin family of matrix proteins, in breast cancer progression. This attention was triggered by the finding that high expression of the α6 subunit in women with breast cancer correlated significantly with reduced survival times (3). In addition, a recent report also revealed a correlation between expression of the β4 subunit and poor prognosis (4). In order to take full advantage of the α6 and β4 subunits as markers for predicting the prognosis of breast cancer, it is necessary to understand mechanistically how these integrins promote aggressive tumor behavior. Until this is established, the full potential of these integrin subunits for diagnosis, or as targets for therapeutic development, will not be known. In previous work we had established that the α6β1 receptor contributes to the growth and survival of breast carcinoma metastases (5). In addition, we had demonstrated that de novo expression of the integrin β4 subunit in colon and breast carcinoma cell lines that lack this integrin subunit increases their invasive potential (6,7). The aims of this career development award were designed to investigate these α6-dependent functions in more molecular detail. In this regard, during the past four years I have made significant progress in accomplishing my goals and as a result, we now have a more detailed understanding of how the a6 integrin receptors contribute to breast cancer.

Body

PI3K is required for the survival of MDA-MB-435 cells (Aim 1). The α6β1 integrin is required for the growth and survival of MDA-MB-435 cells in vivo. To understand the mechanisms involved in the contribution of α6β1 to survival, we established conditions *in vitro* that mimicked the survival differences of the mock and α6β4-ΔCYT transfected cells *in vivo*. Using this assay, we examined the signaling pathways that are involved in α6β1-dependent survival. As shown in Fig. 1, there is a 2.5 fold difference in the amount of apoptosis observed for the α6β4-ΔCYT transfectants when compared with the mock transfectants. Treatment of the mock transfectants with an inhibitor that prevents activation of the mitogen activated protein kinases (MAPK) Erk1 and Erk2 did not alter the survival of these cells. In contrast, treatment of the mock transfectants with wortmannin, an inhibitor of PI3K, resulted in a level of apoptosis that was comparable to that observed for the α6β4-ΔCYT transfectants. These data suggest that α6β1 promotes the survival of MDA-MB-435 cells through activation of a PI3K signaling pathway.

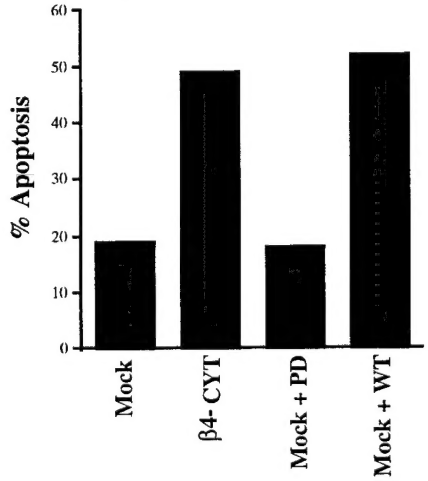

Figure 1: PI3K is required for α6β1-dependent breast carcinoma survival. Inhibition of PI3K by wortmannin (WT) increased the amount of apoptosis observed in the mock transfectant cells to the level observed for the α6β4-ΔCYT transfectants. Inhibition of MEK by PD98059 (PD) did not alter the survival of the mock transfectants.

The α6β1 receptor cooperates with the IGF-1 receptor to activate PI3K and Akt (Aim 1). To examine further the contribution of the α6β1 receptor to the growth and survival of breast carcinoma cells, we compared the ability of the α6β1 and α6β4-ΔCYT receptors to cooperate with IGF-1 to activate PI3K. IGF-1 is a potent survival factor for many cells, including breast carcinoma cells, and the ability of α6β1 to cooperate with IGF-1 would provide a mechanism for the contribution of α6β1 to survival (8). As shown in Figs. 2A and 2B, the α6β4-ΔCYT receptor is impaired in its ability to cooperate with the IGF-1 receptor to activate downstream signals, including PI3K. Significantly more PI3K p85 regulatory subunit was associated with a phosphotyrosine immunoprecipitation after clustering the α6β1 receptor in the presence of IGF-1 than when α6β4-ΔCYT was clustered (Fig. 2B). The Akt serine/threonine kinase is a downstream effector of PI3K that is important for cell survival in many systems (9). As shown in Fig 2C, the α6β1 receptor cooperates with IGF-1 to promote Akt activation to a greater extent than the α5β1 receptor. Taken together, these data suggest that the decreased survival of the α6β4-ΔCYT transfectants could be the result of a decreased ability to respond to survival growth factors such as IGF-1.

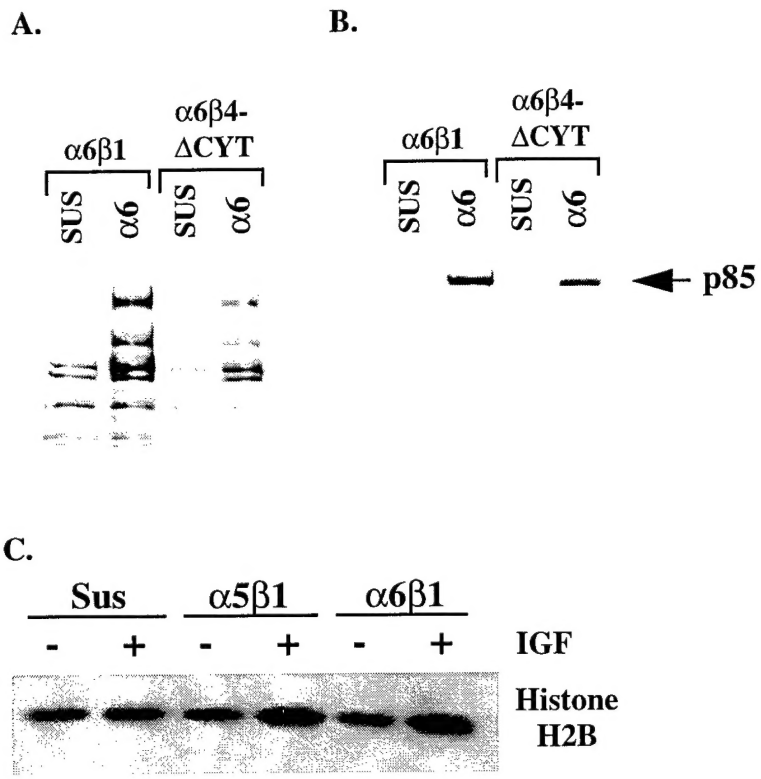

Figure 2: **Cooperative signaling between the α6β1 integrin and the IGF-1R.** MDA-MB-435 transfectants were maintained in suspension or clustered with α6-specific Abs for 30 minutes. IGF-1 (50 ng/ml) was added to the cells for the final 5 minutes of the clustering. Aliquots of cell extracts were immunoprecipitated with pTyr-specific Abs and the immune complexes were immunoblotted with either p-Tyr- (A) or PI3K-specific (p85; B) Abs. C) Cells were treated as described above and the cell extracts were immunoprecipitated with Akt-specific Abs and assayed for their kinase activity using Histone H2B as a substrate. Sus, cells maintained in suspension; α6, cells clustered with an α6-specific Ab; +, incubated with 50 ng/ml IGF-1 for 5 minutes.

Activation of phosphoinositide 3-OH kinase by the α6β4 integrin promotes carcinoma invasion (Aim 2, Cell 1997 *91*:949-960, see Appendix).

In the initial grant application, I presented data showing that expression of the α6β4 integrin promotes invasion. I investigated the mechanism of this invasion promoting function of α6β4 and determined that the α6β4 integrin promotes carcinoma invasion through a preferential and localized targeting of phosphoinositide-3 OH kinase (PI3K) activity. Stable expression of α6β4 increased carcinoma invasion in a PI3K-dependent manner and transient expression of a constitutively active PI3K increased invasion in the absence of α6β4. Ligation of α6β4 stimulated significantly more PI3K activity than ligation of β1 integrins, establishing specificity among integrins for PI3K activation. α6β4-regulated PI3K activity was required for the formation of lamellae, dynamic sites of motility, in carcinoma cells. In addition, I determined that the small G-protein Rac is an essential, but not sufficient,

downstream effector of PI3K for invasion. These studies define a mechanism by which the α6β4 integrin promotes carcinoma invasion and invoke a novel function for PI3K signaling.

PKC-ε regulates the dynamic behavior of actin-rich cell protrusions and is required for the migration and invasion of carcinoma cells (Aim 2, see Appendix).

Cell migration is an essential function of invasive carcinoma. Although many signal transduction pathways have been implicated in the control of cell migration, the mechanisms by which individual signaling molecules regulate the dynamic cytoskeletal events that underlie migration are relatively unknown. Although the protein kinase C (PKC) family has been implicated in migration and invasion, little data exist regarding either isoform specificity or their contribution to the mechanics of migration (10). In this study, we focused on PKC isoforms that are regulated by the lipid products of PI3K, PKC-ε and PKC-ζ, and used a genetic approach to investigate the contribution of these PKC isoforms to carcinoma cell migration. Transient expression of wild type and kinase inactive forms of these PKC isoforms in Clone A colon carcinoma cells revealed that PKC-ε, but not PKC-ζ, is involved in cell migration. The same approach demonstrated that PKC-ε is also essential for the *in vitro* invasion of MDA-MB-435 breast carcinoma cells. Time-lapse videomicroscopy revealed that PKC-ε is essential for carcinoma migration and invasion because it is required for the organization and stabilization of lamellae, and for the retraction of cell processes.

Identification of IRS-1 and IRS-2 as signaling intermediates in the α6β4 integrin-dependent activation of PI3K and promotion of invasion (Aim 2; Mol.Cell.Biol. 2001. *21*:5082-5093, see Appendix).

The involvement of a PI3K-dependent signaling pathway in invasion is supported by other ours and other studies (11) and adds to previous data that have implicated PI3K in tumor promoting functions including transformation (12), cell survival (13, 14), anchorage-independent growth (15), and motility (16). Taken together, these findings support a central role for PI3K and its lipid products in carcinoma progression and highlight the need to investigate in more detail how this pathway is regulated. Therefore, I investigated the signaling pathway by which the α6β4 integrin activates PI3K. Neither the α6 nor the β4 cytoplasmic domain contains the consensus-binding motif for PI3K, pYMXM, indicating that additional proteins are likely to be involved in the activation of this lipid kinase by the α6β4 integrin. I identified Insulin Receptor Substrate-1 (IRS-1) and IRS-2 as signaling intermediates in the activation of PI3K by the α6β4 integrin. IRS-1 and IRS-2 are cytoplasmic adapter proteins that do not contain intrinsic kinase activity but rather function by recruiting proteins to surface receptors where they organize signaling complexes (17). Ligation of the α6β4 receptor promotes tyrosine phosphorylation of IRS-1 and IRS-2 and increases their association with PI3K, as determined by co-immunoprecipitation.

To understand further how α6β4 activates signaling pathways to promote carcinoma invasion, I examined the β4 cytoplasmic domain for potential binding sites for signaling molecules. In this analysis, I identified two immunoreceptor tyrosine-based inhibitory motifs or "ITIM" motifs. The ITIM motifs were initially described in immune cell inhibitory co-receptors and have been shown to be binding sites for the protein phosphatases SHP-1 and SHP-2, and the SH2-containing inositol polyphosphate 5'-phosphatase (SHIP;18). Recruitment of these phosphatases to the β4 cytoplasmic domain could up- or downregulate the signaling functions of the α6β4 receptor by modifying the phosphorylation of β4 itself or other downstream signaling effectors. Through site-directed mutagenesis, I determined that Y1494 in the β4 cytoplasmic domain is required for α6β4-dependent phosphorylation of IRS-2 and activation of PI3K in response to receptor ligation. Most importantly, Y1494 is essential for the ability of the α6β4 integrin to promote carcinoma invasion. Taken together, these results implicate a key role for the IRS proteins in the α6β4-dependent promotion of carcinoma invasion.

Involvement of the src family in the α6β4 integrin-dependent promotion of invasion (Aim 2).

In Specific Aim 2 of the grant, I proposed to determine the role of tyrosine phosphorylation in α6β4-mediated breast carcinoma invasion. An inhibitor of the src family kinases, PP2, was used to investigate the involvement of this family in α6β4-dependent signaling and promotion of invasion. In the presence of PP2, stimulation of tyrosine phosphorylation by α6β4 ligation was markedly diminished (Fig. 3A). In addition, PP2 significantly inhibited the invasion of β4/MDA-MB-435 cells (Fig. 3B). Finally, ligation of the α6β4 integrin promoted src activation (Fig. 3C). Interestingly, inhibition of src family kinases did not prevent IRS-2 phosphorylation and activation of PI3K in response to α6β4 ligation which suggests that the PI3K and src pathways are independent pathways that are both essential for promoting invasion (Fig. 3D). I am continuing these studies to identify the specific src family member(s) that is essential for invasion and how the α6β4 integrin promotes kinase activation.

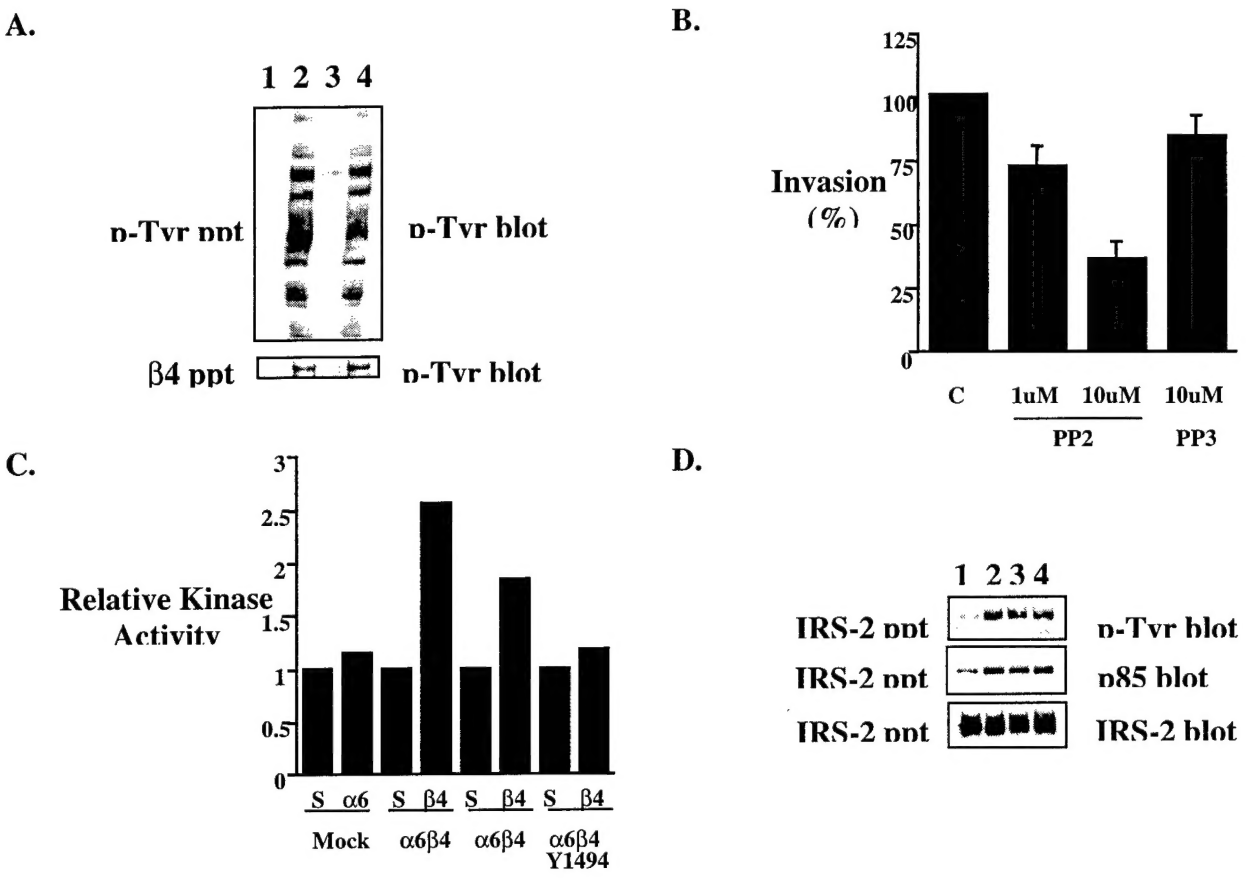

Figure 3: Src-family kinases and α6β4-dependent signaling. A) MDA-MB-435/β4 cells were kept in suspension (1) or clustered with β4-specific Abs in the absence (2) or presence of the src family kinase inhibitor PP2 (10uM;3) or the inactive isomer PP3 (10uM;4). Cell extracts were immunoprecipitated with pTyr- (upper) or β4-specific Abs (lower) and the immune complexes were immunoblotted with pTyr-specific Abs. B) MDA-MB-435/β4 cells were assayed for their ability to invade Matrigel in the absence or presence of PP2 or PP3 at the indicated concentrations. C) MDA-MB-435/mock and β4 cells were kept in suspension (S) or clustered with β4-specific Abs (β4). Cell extracts were immunoprecipitated with src-specific Abs and the immune complexes were assayed for src activity. D) Cells were treated as described in (A) and the cell extracts were immunoprecipitated with IRS-2 specific Abs. The immune complexes were immunoblotted with pTyr, p85, and IRS-2 specific Abs.

Tetracycline-regulated expression of α6β4 in MDA-MB-435 cells (Aim 2).

To evaluate the contribution of α6β4 expression to breast carcinoma invasion and metastasis in vivo, MDA-MB-435 cells that express the β4 subunit under control of a tetracylcine-regulated promoter were established. Stable subclones of MDA-MB-435 parental cells that express the Tet-Off repressor were isolated and screened for low basal expression in the presence of tetracycline and high expression when tetracycline was withdrawn. Two such subclones are shown in in Fig. 4. The β4 subunit was subcloned into the pTRE2 response vector and stably transfected into the #5 and #7 subclones. I am currently screening these subclones and they will be used for *in vivo* assays as described in Aim 2 of this Career Development Award.

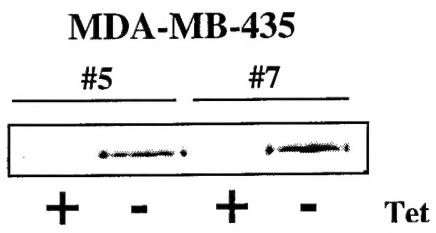

Figure 11: Tetracycline-regulated gene expression in MDA-MB-435 cells. MDA-MB-435 cells were stably transfected with the Clontech Tet-Off repressor. Two individual subclones (#5 and #7) were transiently transfected with an HA-tagged SHP-2 cDNA under the control of a tetracycline-regulated promoter. The cells were incubated for 24 hours in the presence (+) or absence (-) of tetracycline. Cell extracts containing equivalent amounts of total protein were resolved and immunoblotted for the transfected proteins using an HA-specific Ab.

17. Key Research Accomplishments:

- The α6β1 integrin promotes breast carcinoma survival through cooperativity with growth factors to promote PI3K and Akt activation.

- Activation of phosphoinositide 3-OH kinase by the α6β4 integrin promotes carcinoma invasion.

- PKC-epsilon is essential for breast carcinoma invasion and it functions in the organization and stabilization of actin-rich motility structures called lamellae. PKC-epsilon may also contribute to cell motility through its involvement in cell retraction.

- The α6β4 integrin promotes PI3K activation through the IRS proteins IRS-1 and IRS-2.

- The α6β4 integrin activates a src family member which is essential for the ability of this integrin to promote breast carcinoma invasion.

-The src kinase family is not involved in the α6β4-dependent phosphorylation of the IRS proteins.

- Y1494 in the β4 cytoplasmic domain is essential for α6β4-dependent promotion of invasion and activation of PI3K through the IRS proteins.

- MDA-MB-435 cells with tetracycline-regulated expression of β4 have been established.

Reportable Outcomes:

1. Manuscripts

Shaw, L.M., I. Rabinovitz, H. H.-F. Wang, A. Toker, and A.M. Mercurio. 1997. Activation of phosphoinositide 3-OH kinase by the α6β4 integrin promotes carcinoma invasion. Cell. *91*:949-960.

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Mercurio, A.M., R. Bachelder, I. Rabinovitz, K. O'Connor, T. Tani, and L.M. Shaw. 2001. The Metastatic Odyssey: The Integrin Connection. Surgical Oncology Clinics of North America *10*:313-328.

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Mercurio, A.M., R. Bachelder, J. Chung, K.L. O'Connor, I. Rabinovitz, L.M. Shaw and T. Tani. Integrin laminin receptors and breast carcinoma progression. J. Mammary Gland Biol. and Neoplasia. (invited submission)

Shaw, L.M., V. Cenni, A. Toker, and A. Mercurio. PKC-ε regulates the dynamic behavior of actin-rich cell protrusions and is required for the migration and invasion of carcinoma cells. (ECR, in revision)

Jauliac, S., C. Lopez-Rodriquez, L.M. Shaw, A. Rao, and A. Toker. The role of NFAT transcription factors in the regulation of integrin-mediated carcinoma invasion. (Nature, submitted)

2. Abstracts

L.M. Shaw, A. Toker, and A.M. Mercurio. Protein Kinase C-epsilon is involved in the phosphoinositide 3-OH kinase-dependent promotion of carcinoma invasion by the α6β4 integrin. Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215 and Boston Biomedical Research Institute, Boston, MA 02114. (1998 American Society for Cell Biology Annual Meeting)

Leslie M. Shaw, Vittoria Cenni, Alex Toker, and Arthur M. Mercurio. PKC-ε regulates the dynamic behavior of actin-rich cell protrusions and is required for the migration and invasion of carcinoma cells. Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215 (2000 Era of Hope Meeting)

Leslie M. Shaw and Yumiko Honzako. The α6β4 Integrin Promotes Invasion through the IRS-2-dependent activation of PI3K. Division of Cancer Biology and Angiogenesis, Department of Pathology, BIDMC, Boston, MA 02215 (2001 Cell Migration and Invasion Keystone Meeting)

Leslie M. Shaw. Signaling by the α6β4 integrin and carcinoma invasion. Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215 (2001 American Society for Cell Biology Annual Meeting)

3. Funding obtained based on work supported by this Award

NIH-R21 Award: "Integrins and D3 Phosphoinositides in Cancer"
Award Period: April 1, 1999 to March 31, 2001
Harvard Institutional Award: "IRS Function in Breast Cancer"
Award Period: January 1, 2001 to December 31, 2001

NIH-R01 Award: "Insulin Receptor Substrate Function in Breast Cancer"
Award Period: December 1, 2001 to November 30, 2006

4. Employment obtained

Promoted to Assistant Professor of Medicine, Harvard University, May 1999

Established new laboratory in the Department of Pathology at the Beth Israel Deaconess Medical Center.

5. Personnel Supported

Leslie M. Shaw

Conclusions

The overall goal of this career development award was to understand the contribution of the α6 integrins to breast carcinoma progression. My work has resulted in an increased understanding of α6β1-dependent breast carcinoma survival and the important role of PI3K and Akt in this function. I made significant progress toward identifying α6β4-dependent signaling pathways that are involved in promoting breast carcinoma invasion. Activation of PI3K and the downstream effectors Rac and PKC-epsilon are essential for carcinoma invasion. We have identified the IRS proteins, IRS-1 and IRS-2, as intermediates in the activation of PI3K by the α6β4 integrin. In addition, I have identified a tyrosine residue, Y1494, in the β4 cytoplasmic domain that is essential for α6β4-dependent phosphorylation of IRS and promotion of invasion. Finally, I have established an involvement of the src family in α6β4-dependent breast carcinoma invasion. Although I have not completed our proposed in vivo studies on α6β4 function in breast cancer, I have established cell lines with regulated β4 expression to be used in these future experiments. I intend to continue to investigate the mechanism of α6β4 signaling so that we can increase our understanding of how these pathways can be manipulated for therapeutic intervention of breast cancer.

On a personal level, the work performed during this career development award has contributed to my promotion to Assistant Professor of Medicine at Harvard Medical School and the establishment of an independent research program in the Department of Pathology at the Beth Israel Deaconess Medical Center. I have also been approved for funding of an NIH-R01 Award that is focused on breast cancer and I intend to continue my career in the field of breast cancer research.

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Appendices:

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Shaw, L.M., V. Cenni, A. Toker, and A. Mercurio. PKC-ε regulates the dynamic behavior of actin-rich cell protrusions and is required for the migration and invasion of carcinoma cells. (ECR, in revision).

PKC-ε regulates the dynamic behavior of actin-rich cell protrusions and is required for the migration and invasion of carcinoma cells

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Running Title: Involvement of PKC-ε in cell migration

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Abstract: Cell migration is an essential function of invasive carcinoma. Although many signal transduction pathways have been implicated in the control of cell migration, the mechanisms by which individual signaling molecules regulate the dynamic cytoskeletal events that underlie migration are relatively unknown. Although the protein kinase C (PKC) family has been implicated in migration and invasion, little data exist regarding either isoform specificity or their contribution to the mechanics of migration. In this study, we focused on PKC isoforms that are regulated by the lipid products of PI3K, PKC-ε and PKC-ζ, and used a genetic approach to investigate the contribution of these PKC isoforms to carcinoma cell migration. Transient expression of wild type and kinase inactive forms of these PKC isoforms in Clone A colon carcinoma cells revealed that PKC-ε, but not PKC-ζ, is involved in cell migration. The same approach demonstrated that PKC-ε is also essential for the *in vitro* invasion of MDA-MB-435 breast carcinoma cells. Time-lapse videomicroscopy revealed that PKC-ε is essential for carcinoma migration and invasion because it is required for the organization and stabilization of lamellae, and for the retraction of cell processes.

Keywords: PKC-ε, Carcinoma, Migration, Invasion, Actin Cytoskeleton

Introduction

An essential function of invasive carcinoma cells is migration [1]. In contrast to normal epithelial cells, which are polarized and exhibit stable cell-cell and cell-matrix interactions, invasive carcinoma cells often display a mesenchymal phenotype and their ability to migrate contributes to tumor spread [2]. For this reason, the mechanisms that underlie cell migration are critical to understanding carcinoma progression. Recent studies have highlighted the complexity of cell migration with respect to both cytoskeletal dynamics and signal transduction. At the leading edge of migrating cells, polymerization of actin filaments, organization of actin filament networks and the formation of novel adhesive contacts occur [3, 4]. In addition, translocation or actual cell movement usually involves retraction and detachment of cell adhesive contacts at the trailing edge of migrating cells [5]. Knowledge of how these dynamic cytoskeletal events are regulated by extracellular stimuli that promote the migration of carcinoma cells is needed to increase our understanding of this fundamental aspect of invasive cancer.

Many signaling molecules have been implicated in cell migration but the mechanisms by which these molecules influence specific cell functions and cytoskeletal dynamics to facilitate migration are not fully understood. The Protein Kinase C (PKC) family of serine/threonine kinases represent a salient example of such signaling molecules. [6, 7]. The PKC family consists of 11 isoforms that are classified by their mode of regulation: the conventional (α, βI, βII, γ) are regulated by diacylglycerol (DAG) and Ca^{++}; the novel (δ, ε, η, θ) are regulated by DAG but are independent of Ca^{++},; and the atypical (ζ, ι, λ) are independent of both DAG and Ca^{++} [8, 9]. The use of broad specificity pharmacological inhibitors that do not discriminate among the individual isoforms has precluded the identification of specific PKC isoforms that participate in cell migration. In addition, the potential mechanisms by which PKC isoforms function in cell migration need to be established.

To evaluate the contribution of specific PKC family members to cell migration rigorously, we are using a genetic approach. In this study we focused our efforts on PKC-ε because this isoform is activated by phosphoinositide 3-OH kinase (PI3K) and phospholipase C-γ (PLC-γ)

signaling pathways, both of which have been demonstrated to promote cell migration [10]. Moreover, PI3K is essential for carcinoma invasion [11, 12]. We also assessed the involvement of PKC-ζ because the activity of this isoform is regulated by PI3K as well [8]. The results obtained implicate a critical role for PKC-ε in the migration and invasion of carcinoma cells because its activity contributes to the dynamic behavior of actin-rich cell protrusions.

Materials and Methods

Cells Clone A cells were originally isolated from a human poorly differentiated colon adenocarcinoma and were obtained from D. Dexter [13]. Clone A cells were grown in RPMI supplemented with 25mM Hepes (RPMI-H), 10% fetal calf serum, 1% L-Glutamine, and 1% penicillin-streptomycin. The MDA-MB-435 breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository at Georgetown University. The transfection of the MDA-MB-435 cell line with the β4 integrin subunit has been described previously [11]. The MDA-MB-435/β4 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Gibco), 1% L-Glutamine, 1% penicillin-streptomycin (Gibco), and 400ug/ml G418.

cDNA Constructs and Transfections The wild-type and kinase inactive PKC-ζ plasmid constructs have been described previously [14, 15]. The human PKC-ε cDNA was cloned into the mammalian expression vector pcDNA3 (Invitrogen), with the addition of the FLAG epitope (DYKDDDDK) at the N-terminus. The PKC-ε kinase inactive mutant (KI; Lys437Trp) and activation loop T566A (Thr566Ala) mutants were generated by PCR based site-directed mutagenesis using Quickchange (Stratagene). All vector sequences were confirmed by DNA sequencing. Cells were passaged one day prior to transfection and plated at 80% confluency. Cells were co-transfected with either 1ug pCS2-(n)β-Gal or Green Fluorescent Protein (GFP;Clontech) and the cDNAs specified in the Figure Legends using Lipofectamine (Gibco) according to manufacturer's instructions. Cells were harvested 24 hrs after transfection for

experiments. To confirm the expression of the transfected proteins, cell extracts from the transfected cells were immunoprecipitated with a FLAG-specific mAb (M2;Sigma). The immunoprecipitates were resolved by electrophoresis on SDS-polyacrylamide gels (8%) and transferred to nitrocellulose. The tagged proteins were detected by immunoblotting with PKC-ε (Santa Cruz) and –ζ (Santa Cruz) specific antibodies.

Kinase Assays Protein kinase activity of the PKC-ε and PKC-ζ proteins was assayed in transiently transfected Clone A and MDA-MB-435/β4 cells. Cells were transfected as described above and then maintained in growth medium containing 10% FBS. After washing with ice-cold 1X PBS, the cell monolayers were lysed in a 1% NP-40 lysis buffer as described previously **[14]**. After clearing the lysate at 14,000rpm, 5% of the total cell lysate was boiled in SDS sample buffer and the rest of the lysate was snap-frozen in liquid N_2 and stored at -70°C. Relative expression levels of the PKC-ε and PKC-ζ proteins were detected by SDS PAGE followed by immunoblotting with PKC-ε- (Transduction labs) and PKC-ζ- (Santa Cruz) specific antibodies. The immunoblots were exposed to a Biorad Molecular Imager chemiluminescence screen and the relative amounts of each of the PKC proteins were quantitated. Cell lysates containing equivalent amounts of PKC protein were immunoprecipitated with the FLAG-specific antibody (M2, Sigma) and a 50/50 mix of Protein A/G (Santa Cruz) for 3 hours at 4°C. The immunoprecipitates were washed stringently and subjected to an *in vitro* kinase assay using Histone H2B as a substrate (Boehringer Mannheim) as described **[14]**. Incorporation of 32P-[ATP] was detected by autoradiography.

Cell Migration and Invasion Assays Haptotactic migration assays were performed using laminin as described previously [16]. After a 5 hr incubation, the cells that had not migrated were removed from the upper face of the filters using cotton swabs. The cells that had migrated to the lower surface of the filters were fixed in 4% formaldehyde for 30 minutes and then stained with PBS containing 1mg/ml Bluo-gal (Boehringer Mannheim), 2mM $MgCl_2$, 5mM Potassium

Ferrocyanide, and 5mM Potassium Ferricyanide. Migration was quantitated by counting using brightfield optics with a Nikon Diaphot microscope. All of the β-galactosidase positive cells were counted for each well.

Matrigel invasion assays were performed as described previously [11]. After 5 hrs, the cells that had invaded to the lower surface of the filters were fixed, stained, and counted as described above for the migration assays.

Indirect Immunofluorescence Microscopy Clone A cells that had been transiently transfected with the PKC constructs were trypsinized, washed two times with RPMI containing 0.1% BSA, and plated onto coverslips that had been coated overnight with 20ug of EHS laminin. After a 45 minute incubation, the medium was removed and the cells were fixed by the addition of a 10mM Pipes buffer, pH 6.8, containing 4% paraformaldehyde, 100mM KCl, 2mM EGTA, 2mM $MgCl_2$, and 7% sucrose. After washing three times with PBS, the cells were permeabilized for 2 minutes by the addition of the same buffer lacking the paraformaldehyde and containing 0.05% Triton-X-100. The coverslips were incubated for 30 minutes in blocking buffer (PBS containing 3% BSA and 1% goat serum) and then for 30 minutes in blocking buffer containing a 1:200 dilution of anti-FLAG mAb. After three 10 minute washes in PBS, the coverslips were incubated for an additional 30 minutes in blocking buffer containing a 1:200 dilution of Cy2-conjugated goat-anti-mouse IgG (Jackson Laboratories). The cells were visualized by confocal microscopy (Bio-Rad).

Video Microscopy Cells that had been transiently co-transfected with GFP and the PKC cDNA constructs were plated on laminin coated coverslips in 60mm culture dishes. After 15 minutes at 37°C in a humidified atmosphere, the dishes were sealed with parafilm and then placed on a microscope stage heated to 37°C. An inverted microscope (model Diaphot 300; Nikon, Inc., Melville, NY) with phase contrast optics was used for image analysis. This microscope was connected to a CCD camera (Dage-MTI, Michigan City, IN), a framegrabber (Scion, Frederick,

MD), and a G3 Power Macintosh computer to capture the images. GFP positive cells were identified and then the images were collected at 1 minute intervals for 30-60 minutes.

Results

Involvement of PKC-ε in carcinoma cell migration

Clone A colon carcinoma cells were used initially to investigate the involvement of specific PKC isoforms in cell migration. These cells exhibit rapid, chemokinetic migration on laminin-1 that is characterized by the formation of fan-shaped lamellae [17]. To examine the contribution of PKC-ε and PKC-ζ to the migration of Clone A cells, wild-type PKC-ε (WT-ε) and PKC-ζ (WT-ζ), as well as kinase-inactive mutants of PKC-ε (KI-ε) and PKC-ζ (KI-ζ) were expressed transiently in these cells. Expression of the PKC isoforms was confirmed by immunoblotting using FLAG-specific antibodies (Fig. 1A, lower panel). In addition, *in vitro* kinase assays were performed to assess the relative activity of the PKC proteins in Clone A cells. As shown in Fig 1B, both the KI-ε and -ζ proteins exhibited decreased kinase activity relative to their corresponding WT-proteins.

The migration of clone A cells that expressed the various PKC proteins toward a laminin gradient was assessed using a modified Boyden chamber. As shown in Fig. 1A, expression of WT-ε increased the migration of Clone A cells by 2.5 fold. An increase in cell migration was not observed in cells that expressed WT-ζ. In the converse experiment, expression of dominant-negative KI-ε, but not KI-ζ, inhibited the migration of Clone A cells by 80% (Fig. 1A). Taken together, these results suggest that PKC-ε is essential for the migration of Clone A cells.

Localization of PKC-ε to membrane ruffles and lamellae

To obtain additional insight into the function of PKC-ε in cell migration, we examined the morphology of Clone A cells that had adhered to laminin-1 after transfection with the WT and KI-PKC constructs, and we examined the spatial distribution of the transfected PKC proteins using indirect immunofluorescence microscopy. Cells that expressed the PKC proteins were fixed after

attachment to laminin-1 for 45 minutes and the exogenous PKC isoforms were detected using a FLAG-specific mAb. Two representative cells from each transfection are shown in Fig. 2. The morphology of Clone A cells that expressed WT-ε did not differ significantly from the morphology of cells transfected with a vector alone (data not shown). These cells were polarized and displayed broad lamellae with extensive membrane ruffling (Fig. 2A,B and Fig. 3A). Interestingly, PKC-ε was concentrated in these membrane ruffles at the leading edge of lamellae (Fig. 2A,B;arrow). PKC-ε was also expressed throughout the cytoplasm and it was absent from the nucleus. The morphology of the Clone A cells that expressed WT-ζ was similar to the morphology of the cells expressing WT-ε and the WT-ζ protein was also distributed diffusely throughout the cytoplasm. Although the WT-ζ protein was observed in membrane ruffles, the concentration of this PKC isoform in these structures was much lower than that observed for the WT-ε protein. (Fig 2F; arrow).

Clone A cells that expressed dominant negative KI-ε attached to laminin-1 to the same extent as cells that expressed WT-ε (data not shown), an observation that discounts a role for PKC-ε in regulating cell adhesion. However, cells that expressed KI-ε displayed a disorganized morphology as evidenced by their lack of a polarized leading edge and well-developed lamellae (Fig. 2C,D). Moreover, irregular membrane protrusions were observed and many long, filopodial-like extensions were also present. The KI-ε protein was distributed throughout the cytoplasm in a grainy, punctate manner and displayed some concentration at the membrane edge (Fig. 2C, arrow). Expression of the mutant PKC-ε was also observed in the filopodial-like extensions. The morphology of cells expressing KI-ζ and the localization of the KI-ζ protein were similar to that observed for the WT-ζ transfectants (Fig. 2G,H).

PKC-ε is required for organization of lamellae and retraction of cell processes

The localization of PKC-ε to membrane ruffles in lamellae and the lack of these organized structures in the cells expressing the dominant negative PKC-ε suggested that this PKC isoform functions in the formation of these actin-rich structures. To explore further the contribution of

PKC-ε to lamellar function, we analyzed the movement and behavior of cells using time-lapse videomicroscopy. After attachment to laminin, Clone A cells extend filopodia, which serve as guides for the extension of lamellae [17]. Subsequently, the cell body translocates in the direction of the developed lamellae. At the trailing edge of the cell, retraction fibers are formed as the cell body moves forward and these fibers eventually detach from the matrix. This pattern of movement was observed for Clone A cells that expressed WT-ε. As shown in Fig. 3A, Clone A cells that expressed WT-ε formed organized, leading edge lamellae with active membrane ruffles and these cells migrated in the direction of lamellar formation (white arrow). As the cell moved, retraction fibers were formed at the trailing edge of the cell (Fig. 3A, black arrow).

The dynamic behavior of Clone A cells on laminin that expressed the dominant negative KI-ε protein, as assessed by videomicroscopy, revealed several important details about the function of PKC-ε. Although these cells were capable of forming lamellae (Fig. 3B;arrowhead), the lamellae formed were significantly smaller and not organized in a polarized manner as observed in the WT-ε transfectants. Moreover, the lamellae that formed in the KI-ε transfectants were less stable and collapsed more rapidly than the lamellae that formed in the untransfected and WT-ε transfected cells. Interestingly, cells that expressed the KI-ε construct also exhibited defects in their retraction of cell processes. When the small lamellae collapsed in these cells, thin retraction fibers were evident. Over time, many of these extensions formed, which resulted in the "filopodial-like" appearance that was observed in the fixed cells. Although filopodia were identified in the KI-ε expressing cells, most of these extensions were determined to be retraction fibers from the videomicroscopic analysis. Interestingly, small lamellae extended along these retraction fibers, similar to the formation of lamellae in the direction of filopodia as described above (arrow; Fig. 3B; 17). Collectively, the data obtained from time-lapse videomicroscopy indicate that PKC-ε contributes to the organization and stabilization of lamellae. In addition, PKC-ε activity is required for efficient retraction of cell processes.

PKC-ε functions in carcinoma invasion

The data we obtained on the involvement of PKC-ε in the migration of Clone A cells provided a rationale for assessing the importance of this PKC isoform in an *in vitro* model of carcinoma invasion. In previous work, we established that expression of the α6β4 integrin in MDA-MB-435 breast carcinoma cells stimulates their invasive potential by a mechanism that involves activation of PI3K and Rac, signaling events that culminate in the formation and stabilization of lamellae [11]. To assess the relative contribution of PKC-ε to invasion, we expressed the PKC-ε and -ζ cDNAs in the MDA-MB-435 cells that also expressed the α6β4 integrin (MDA-MB-435/β4). An additional dominant negative mutant of PKC-ε, T566A-ε, was also used. This cDNA contains a mutation at threonine 566 in the activation loop that does not permit activation of this PKC isoform. Expression of the PKC proteins after transient transfection into the MDA-MB-435/β4 cells was confirmed by immunoblotting using a FLAG-specific mAb (Fig. 4A; lower panel). In addition, *in vitro* kinase assays were performed to assess the relative activity of the PKC constructs in the MDA-MB-435/β4 cells (Fig. 4B). As was observed with Clone A cells, the activity of the KI-ε and -ζ proteins was diminished in comparison to their respective wild type proteins. The activity of the T566A-ε mutant protein was also decreased confirming that this mutant functions as a dominant negative PKC-ε protein as well.

The ability of the MDA-MB-435/β4 cells that expressed the different PKC proteins to invade through a Matrigel-coated filter was examined. As shown in Figure 4A, expression of either the WT-ε, WT-ζ, or KI-ζ PKC proteins did not alter the invasion of MDA-MB-435/β4 cells significantly in comparison to control cells. In contrast, expression of either of the dominant negative PKC-ε mutants inhibited invasion by 50-60%. These data implicate an essential role for PKC-ε in the invasion of MDA-MB-435/β4 cells and, together with the data obtained for the Clone A cells, support a general role for PKC-ε in carcinoma migration and invasion.

Interestingly, expression of WT-ε in Clone A cells increased their migration by 2.5 fold but did not significantly increase the invasion of the MDA-MB-435/β4 cells. This discrepancy could be explained by differences in the levels of endogenous PKC-ε expression and activity. As shown in Fig. 4C, a relatively low level of expression of PKC-ε was detected in Clone A cells in comparison

to the level detected in the MDA-MB-435/β4 cells. If the levels of PKC-ε detected in the MDA-MB-435/β4 cells are optimal for function, expression of endogenous WT-ε would not increase motility further.

Discussion

In this report, we implicate an important role for PKC-ε in the migration and invasion of carcinoma cells and define the contribution of this kinase to the mechanics of migration. Our use of a genetic approach allowed a rigorous assessment of the function of specific PKC isoforms in the motile behavior of two-well studied carcinoma cell lines, Clone A colon carcinoma cells and MDA-MB-435 breast carcinoma cells. Specifically, the data we obtained indicate that PKC-ε is essential for carcinoma migration and invasion because it contributes to the organization and stabilization of actin-rich protrusions termed lamellae that are essential for cell movement. Interestingly, the contribution of PKC-ε to lamellae stabilization provides a mechanistic explanation for the previously reported involvement of this PKC isoform in integrin-dependent cell spreading [18]. In addition, our findings indicate that the activity of PKC-ε is also required for the efficient retraction of cell processes. Although we excluded the involvement of PKC-ζ in cell migration in the model systems used in this study, it is possible that other PKC family isoforms such as PKC-α, δ, and θ cooperate with PKC-ε to promote migration [19-22]. Clearly, a careful genetic analysis of the function of all of the PKC-isoforms is needed before a complete understanding of the contribution of the PKC family to carcinoma motility and invasion is established.

The ability of a kinase inactive mutant of PKC-ε to inhibit cell motility indicates that the activity of PKC-ε is essential for its function in cell migration and that substrate(s) of this kinase are involved, either directly or indirectly, in regulating the morphological events that underlie cell movement. A number of regulatory and cytoskeletal proteins are required for lamellae organization including proteins that nucleate actin polymerization, promote actin filament cross-linking and promote filament disassembly [23]. Although much is known about the functions of these actin-modifying proteins, the mechanisms for how each of these proteins are regulated by the stimuli that

promote lamellae formation have not been elaborated fully and need to be investigated further. To date, none of the actin regulatory or structural proteins that are involved in lamellae formation have been reported to be phosphorylated directly by PKC-ε, although some, including ABP-280 and MARCKS, can be phosphorylated in a PKC-dependent manner [24, 25]. Unfortunately, the isoform specificity of this phosphorylation has not been determined. An alternative possibility is that PKC-ε promotes lamellae organization through an indirect mechanism. In this regard, tyrosine phosphorylation of the docking protein p130Cas, which has been implicated as a "molecular switch" for cell migration, is promoted by PKC-ε activity [26-28]. Although the actual function of p130Cas in cell migration has not been determined, it is likely that p130Cas regulates the organization and localization of signaling complexes that are required for cell migration, and we suggest that such complexes may be regulated by PKC-ε.

The inability of Clone A cells that lack PKC-ε function to retract cell processes efficiently suggests that PKC-ε could be involved in regulating adhesive strength, or interestingly, in cell contraction [5]. Tension is required for a migrating cell to disrupt adhesive contacts and retract cell processes and this tension is generated by contraction of the cell body [5]. The finding that PKC-ε may be involved in cell contraction is intriguing in light of the established role for PKC-ε in smooth muscle contraction [29]. The Ca^{++}-independent contraction of smooth muscles is mediated by PKC-ε-dependent phosphorylation of the F-actin binding protein calponin [29, 30]. In many non-smooth muscle cells, PKC-ε binds to F-actin when it is activated and this translocation from the cytosol to the actin cytoskeleton localizes the kinase to the appropriate site for the phosphorylation of calponin [31, 32]. Although calponin is expressed primarily in muscle cells, a non-smooth muscle isoform of calponin, calponin h2, has a more ubiquitous expression pattern [33, 34]. This novel calponin isoform could play a role, downstream of PKC-ε, in the actin/myosin contractions that are required for cell migration.

PKC-ε is a novel PKC family member that is activated by stimuli that promote PLC-γ-dependent hydrolysis of PI-4,5-P_2 to produce DAG [8]. In addition, PKC-ε associates with

phosphoinositide-dependent kinase-1 (PDK-1), a serine/threonine kinase that is dependent upon PI3K for function. [35-37]. Several of the PKC isoforms including PKC-βII, -ζ, -δ, and -ε, are phosphorylated by PDK-1 and this phosphorylation is essential for their activation [14, 38, 39] (A. Toker, unpublished data). A role for PLC-γ in promoting PKC-ε-dependent migration is supported by the fact that stimulation of chemotaxis by a number of growth factors including PDGF-β and EGF is dependent upon PLC-γ activation [40, 41]. However, to complicate matters, this growth factor-dependent chemotaxis can also require activation of PI3K [40]. Although it remains an open question at this time, it is highly likely that PLC-γ and PI3K signaling pathways cooperate to activate PKC-ε to promote cell migration. With regard to the involvement of PI3K in PKC-ε activation, we recently demonstrated that the PI3K-dependent chemoinvasion of carcinoma cells requires the small GTPase Rac, but that other PI3K effectors are also essential because Rac is not sufficient to promote invasion on its own [11]. A model could be proposed in which PKC-ε cooperates with Rac, downstream of PI3K, to promote cell migration. Rac activation would promote the initial formation of lamellae whereas PKC-ε would participate in their maintenance and stability.

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Abbreviations

PKC, Protein Kinase C; DAG, diacylglycerol; PI3K, Phosphoinositide 3-OH kinase; PLC-γ, phospholipase C-γ; PDK-1. Phosphoinositide-dependent kinase-1.

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Figure Legends

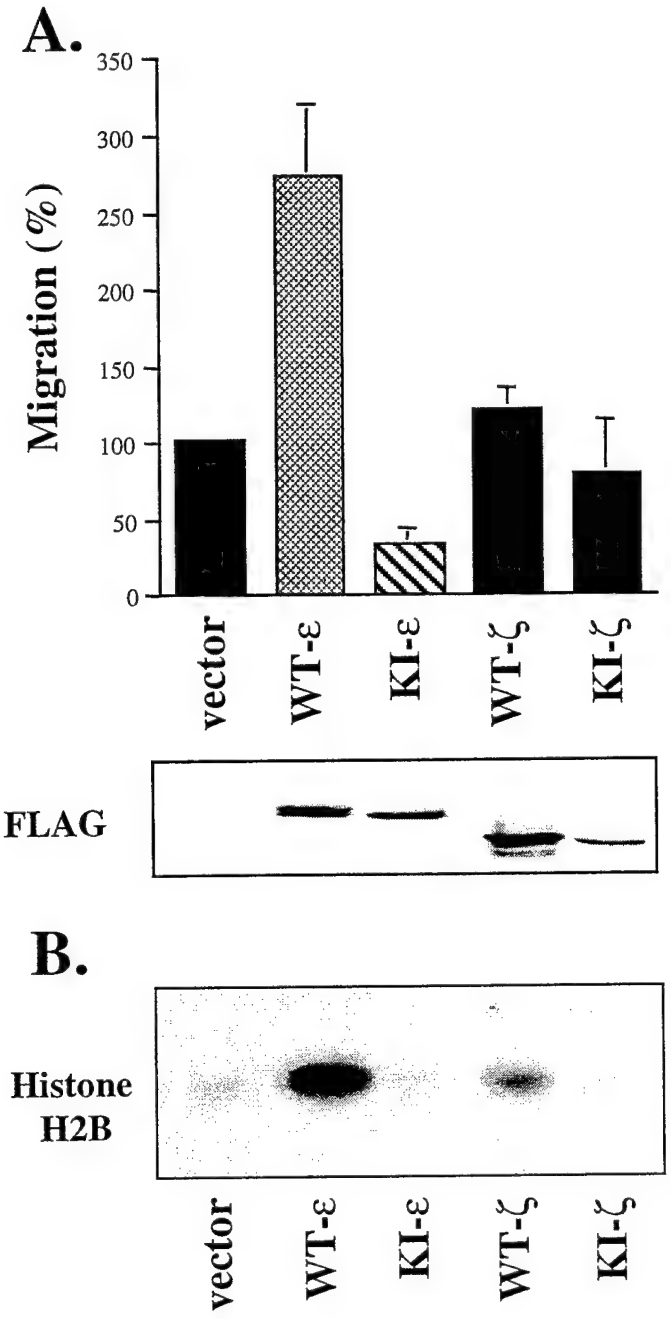
Figure 1: **Analysis of PKC involvement in migration of Clone A cells by transient transfection.** A) Clone A cells were assayed for their ability to migrate toward a laminin-1 gradient after transient transfection with PKC cDNAs. Cells were transiently transfected with 1ug pCS2-(n)β-gal and 4ug of either the vector alone, WT-ε, KI-ε, WT-ζ, or KI-ζ. Transfected cells (10^5) were added to the upper wells of Transwell chambers and medium containing 15ug/ml of laminin-1 was added to the bottom wells. After 5 hrs at 37°C, the cells that had not migrated were removed and the cells that had migrated to the lower surface of the filters were fixed, stained, and quantitated as described in Materials and Methods. Expression of the transfected proteins was detected by immunoprecipitating with a FLAG-specific mAb and then immunoblotting with PKC-isoform-specific antibodies (lower panel). The data shown are the mean values (±SEM) of six (ε) or four (ζ) experiments done in duplicate. B) *In vitro* kinase assays were performed to assess the relative activity of the PKC proteins in the Clone A cells. PKC activity was assayed on a FLAG immunoprecipitate using Histone H2B as a substrate. WT, wild type; KI, kinase inactive.

Figure 2: **Morphological analysis of Clone A cells transfected with PKC-ε and -ζ.** Clone A cells that had been transiently transfected with the indicated PKC cDNAs were allowed to adhere to laminin-1 for 45 minutes. The cells were fixed and stained for the expression of the transfected PKC proteins using a FLAG-specific Ab followed by a Cy2-conjugated anti-mouse antibody as described in Materials and Methods. The cells were visualized by confocal microscopy (Bio-Rad). Two representative images from each transfection are shown. A and B) Cells transfected with WT-ε display broad, polarized lamellae with extensive membrane ruffles. PKC-ε is concentrated in the membrane ruffles (arrows). C and D) Cells transfected with KI-ε lack polarized lamellae and display numerous filipodial-like structures (arrowhead). KI-ε is concentrated at the membrane edge (arrows). E and F) Cells transfected with WT-ζ display broad, polarized lamellae but PKC-ζ is not concentrated in the membrane ruffles (arrow). G and H) Cells transfected with KI-ζ display a similar morphology to the WT-ζ expressing cells. Bars, 10uM.

Figure 3: **Videomicroscopic analysis of Clone A cells expressing WT- and KI-ε on laminin-1.** Clone A cells were co-transfected with a vector containing GFP and a vector containing either WT- or KI-ε. The transfected cells were plated on a laminin-1 substrate and GFP positive cells were analysed by time-lapse videomicroscopy. Images were obtained using a Nikon Diaphot 300 inverted microscope with phase contrast optics. This microscope was connected to a CCD camera (Dage-MTI), a frame-grabber (Scion) and a G3 Power Macintosh computer to capture the images. A) Clone A cells expressing WT-ε form polarized, leading edge lamellae with membrane ruffles (white arrow) and these cells migrate in the direction of lamellae formation. B) Clone A cells expressing KI-ε form small, non-polarized lamellae that are less stable than the lamellae formed by the WT-expressing Clone A cells (arrowhead). The cells expressing KI-ε also exhibit defects in the retraction of cell processes (arrow).

Figure 4: **Analysis of PKC involvement in invasion of MDA-MB-435/β4 cells by transient transfection.** A) MDA-MB-435/β4 cells were assayed for their ability to invade Matrigel after transient transfection with PKC cDNAs. Cells were transiently transfected with 1ug pCS2-(n)β-gal and 4ug of either the vector alone, WT-ε, KI-ε, T566A-ε, WT-ζ, or KI- ζ. Transfected cells (10^5) were added to the upper wells of Matrigel-coated Transwell chambers and conditioned 3T3-media was added to the bottom wells. After 5 hrs at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Materials and Methods. Expression of the transfected proteins was detected by immunoprecipitating with a FLAG-specific mAb and then immunoblotting with PKC-isoform-specific antibodies (lower panel). The data shown are the mean values (±SEM) of six (WT-ε and KI-ε) or four (T566A-ε, WT-ζ and KI-ζ) experiments done in triplicate. WT, wild type; KI, kinase inactive. B) In vitro kinase assays were performed to assess the relative activity of the PKC proteins in the MDA-MB-435/β4 cells. PKC activity was assayed on a FLAG immunoprecipitate using Histone H2B as a substrate. C) Total cell extracts

from Clone A and MDA-MB-435/β4 cells containing equivalent amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with PKC-ε specific antibodies. A low level of PKC-ε expression was detected in the Clone A cells in comparison to the level of PKC-ε protein detected in the MDA-MB-435/β4 cells.


A.
Migration (%)
350
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250
200
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50
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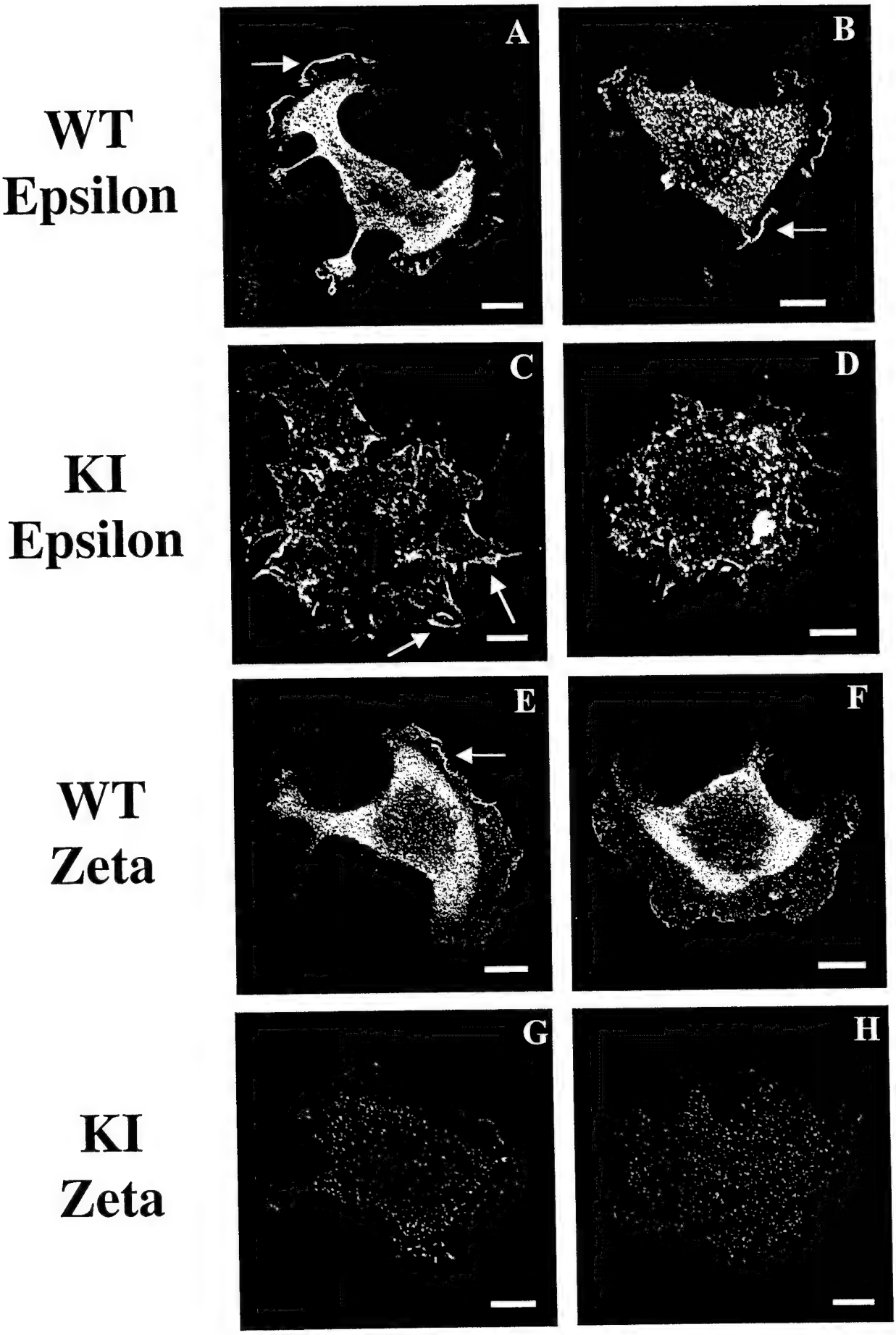
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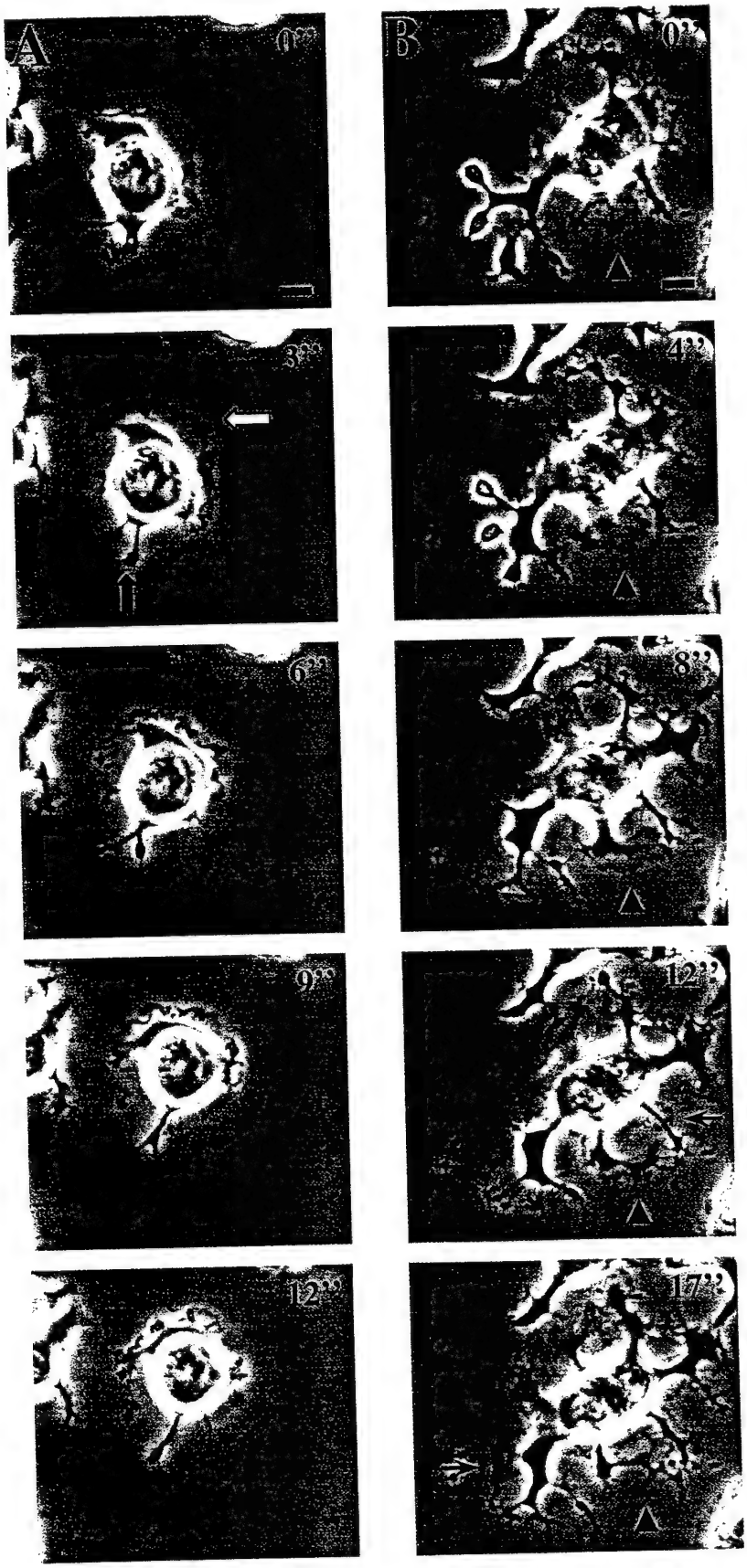
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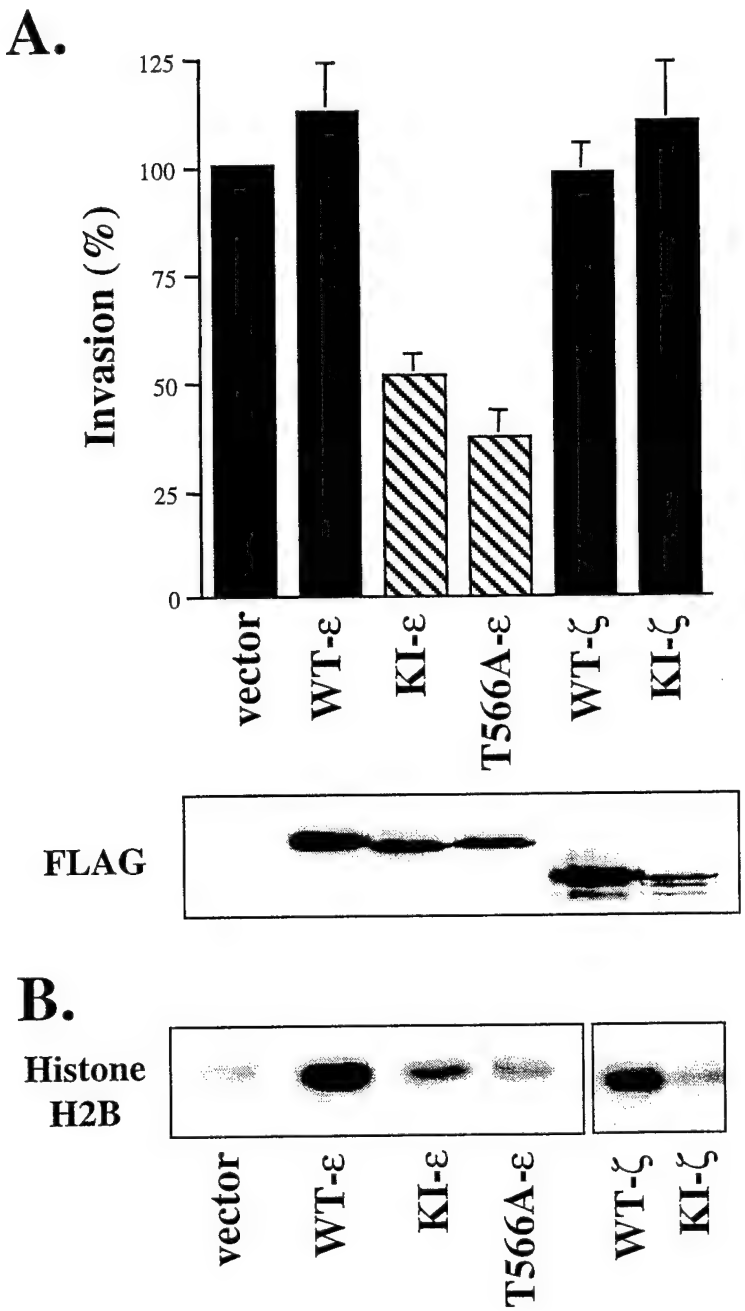
A.
125
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75
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Invasion (%)
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WT-ε
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B.
Histone
H2B
vector
WT-ε
KI-ε
T566A-ε
WT-ζ
KI-ζ
C.
Cl A 435/β4
PKCε →

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Integrin Function in Breast Carcinoma Progression[1]

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The differentiation and function of mammary epithelial cells is dependent upon the combined action of growth factor/hormone receptors and integrin receptors, which act in concert to control the signals required for normal cell function. It is now becoming clear that integrin receptors also contribute to carcinoma cell behavior and that alterations in expression and function during transformation have a large impact on breast carcinoma progression. The focus of this review is to discuss integrin-dependent functions that can be manipulated as targets for the therapeutic intervention of breast cancer. A combination of correlative and mechanistic studies have contributed to the identification of specific integrin receptors, namely $\alpha 2\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$, implicated in breast carcinoma progression. Although this field is still emerging and much remains to be learned, potential integrin-dependent signaling targets have been identified.

KEY WORDS: Integrin receptors; invasion; breast carcinoma; phosphoinositide 3-OH kinase; extracellular matrix.

INTRODUCTION

The integrins are a family of cell-cell and cell-extracellular matrix receptors that play important roles in many cellular functions (1). These $\alpha\beta$ heterodimers interact with their extracellular ligands as well as with the intracellular cytoskeleton to relay information in both directions across the plasma membrane (1). In addition to their adhesive functions, integrins can activate intracellular signaling pathways that regulate growth, differentiation, cell motility, and gene expression (2,3). Many of the signaling pathways that have been characterized for growth factor receptors can also be stimulated by integrin receptor engagement. In fact, these two receptor systems may act in concert to elicit the specific signals that are required for proper cell function (4). Therefore, it is not surprising that alterations in integrin expression and function during transformation are likely to have a large impact on carcinoma progression (5,6). The goal of this review is to identify specific integrins and integrin-regulated signaling pathways that can be manipulated as targets for therapeutic intervention of breast cancer. As will be evident, this is an emerging area and much remains to be learned about integrin function in breast carcinoma, as well as other carcinomas. However, the available data highlight the potential importance of integrins in breast cancer and, hopefully, the themes that emerge from this review will provide a framework upon which future investigations can continue to build.

INTEGRIN EXPRESSION IN THE NORMAL BREAST

An understanding of integrin expression and function in the normal breast provides a foundation to study these parameters in breast cancer progression. It should be noted at the outset that there are several caveats to using integrin expression data to develop models by which integrins contribute to cellular function. Most

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importantly, the expression of a particular integrin does not confirm that this receptor is functional. There are numerous examples of inactive integrins that require exogenous stimuli to activate binding to their ligands (1). Furthermore, we do not at present know what level of expression is sufficient for an integrin receptor to alter cellular function. Nonetheless, identifying the repertoire of integrins that are expressed in a given tissue is a starting point from which to develop more mechanistic studies. A number of investigators have examined the expression of integrin subunits in the normal mammary gland using immunohistochemistry and a general concensus has emerged (Table I).

The mammary epithelium is composed of two major cell types, myoepithelial and luminal epithelial cells. These cells have unique functions that are reflected in their expression of integrin receptors. Myoepithelial cells of the breast express the α1, α2, α3, α5, αv, α6, β1, and β4 subunits (7–10). These integrin subunits are polarized primarily at the basal surface where interactions with their extracellular matrix ligands occur. Luminal epithelial cells express a similar panel of integrin subunits, albeit at lower levels than observed for myoepithelial cells (8,9). Interestingly, the α1β1 integrin, which is expressed primarily in smooth muscle cells, is expressed only in myoepithelial cells of the breast and reflects their contractile nature (11). The α1 subunit is expressed at the onset of myoepithelial cell differentiation and its expression is maintained throughout development and in the adult. Therefore, the α1β1 integrin may contribute to the maintenance of the smooth muscle differentiation of this specialized epithelial layer (11). The fact that breast carcinomas rarely arise from the myoepithelium and the observation that α1β1 is not expressed in most breast carcinomas discount a role for this integrin in the pathogenesis of breast cancer.

Myoepithelial cells also express the α6β4 integrin, a receptor for members of the laminin family of extracellular matrix proteins, at much higher levels than luminal epithelial cells (8,9). The α6β4 integrin is localized on the basal surface of myoepithelial cells within discreet, adhesive structures, termed hemidesmosomes, which are responsible for the stable adhesion of epithelial monolayers to the underlying basement membrane (12–16). In these structures, the α6β4 integrin interacts with the cytokeratin cytoskeleton (15,16). Although hemidesmosomes were originally characterized in stratified epithelia, both myoepithelial and luminal mammary epithelial cells form hemidesmosomes at sites of attachment to the basement membrane (17). In the luminal cell layer, a higher level of β4 expression is found in the aveolar luminal cells than in the ductal luminal cells, a pattern that reflects the more frequent contact of these cells with the basement membrane (11).

INTEGRIN FUNCTION IN THE NORMAL BREAST

The differentiation and function of the mammary gland is dependent upon the concerted action of both soluble factors, such as hormones and growth factors, and insoluble factors, such as extracellular matrix proteins (18,19). A classic example is the requirement *in vitro* of certain normal mammary cells to be grown in the presence of laminin-1 and lactogenic hormones to differentiate and express milk protein genes (20). The dependence of these cells on laminin is mediated through β1 integrin receptors (21). Detailed studes have been performed to examine this cooperative effect. One mechanism by which matrix interactions regulate milk protein synthesis is indirect and it

Table I. Summary of Integrin Expression and Function

Integrin receptor	Myoepithelial	Luminal epithelial	Invasive carcinoma	Proposed function in carcinoma
α1β1	++	−	−	Not expressed
α2β1	++	+	+/−	Regulates epithelial differentiation
α3β1	++	+	+/−	?
α5β1	+	+	+/−	?
α6β1	+++	+	+/++	Promotes growth and survival
αvβ?	+	+/−	+	? (The association of αv with β1, β3, β5, or β6 has not been clearly established)
α6β4	+++	+	+/++	Promotes invasion

involves the transcriptional control of inhibitors of milk protein genes. The expression of TGF-β1 and TGF-α, which inhibit the transcription of β-casein and whey acidic protein, is strongly inhibited by integrin-dependent interactions with the extracellular matrix (22,23). Another mechanism of cooperativity is shown in prolactin stimulation of transcription of β-lactoglobulin mRNA through activation of the transcription factor, Stat5. In the absence of laminin-1, Stat5 binding activity for the β-lactoglobulin gene is not induced (24). In fact, prolactin does not induce phosphorylation of its receptor or the associated signaling kinase, Jak2, unless the cells interact with laminin-1, an effect that may be controlled through integrin regulation of a protein tyrosine phosphatase(s) (25). These examples of cooperativity between integrins and growth factors/hormones emphasize the contribution of integrin receptors to the normal biology of mammary epithelial cells.

It is well documented that disregulation of growth factor receptor signaling pathways, either through changes in expression levels or oncogenic activation, contributes to breast cancer progression (26). Given the complex nature of the relationship between these two receptor systems, it is not surprising, therefore, that alterations in either the expression or function of integrin receptors also disturb the balance required for normal differentiation and promote tumor progression.

INTEGRIN EXPRESSION AND FUNCTION IN BREAST CARCINOMA

The contributions of integrin receptors to breast carcinoma progression have been investigated in numerous immunohistochemical studies, as well as in more recent mechanistic studies (Table I). When reviewing the data on integrin expression in breast cancer it is important to keep in mind that breast cancer arises primarily from luminal epithelial cells and very rarely from cells of the myoepithelial lineage (27). In the normal breast, intense staining for many of the integrin subunits is seen concentrated at the basement membrane in the myoepithelial layer, as mentioned above (28). In invasive carcinomas, this cell layer is most often absent and the expression of integrin subunits on the surface of carcinoma cells is diffuse. This staining pattern has led to the erroneous assumption that there is an overall decrease, or absence, of integrin expression in breast carcinoma. Although the expression of some integrin subunits is decreased, it is clear from many *in vitro* and *in vivo* studies that integrin receptors are expressed in breast adenocarcinomas and that they contribute significantly to the pathobiology of breast cancer. The integrin receptors that have been most clearly implicated in breast cancer progression are α2β1 and the α6 integrins (α6β1 and α6β4).

THE α2β1 INTEGRIN

The α2β1 integrin is a dual-specificity receptor that recognizes collagens I and IV and members of the laminin family (1). The α2 subunit is expressed basally as well as laterally in luminal epithielial cells (8,10). In the latter location, it may contribute to cell-cell interactions as it has been suggested to do in other epithelial cell types (29). The expression of α2β1 is maintained in benign breast lesions such as fibrocystic disease or fibroadenomas (8,10). However, the expression level of α2β1 decreases with the differentiation status of breast adenocarcinomas. Specifically, poorly differentiated adenocarcinomas express very low, or undetectable levels of α2β1 while moderately differentiated adenocarcinomas express intermediate levels (10,30). These correlative studies suggest that the α2β1 integrin is important for maintaining differentiation and controlling proliferation of the breast epithelium and that its loss is essential for the progression to invasive carcinoma.

The possibility that α2β1 contributes to mammary epithelial differentiation has been addressed more vigorously by studies in which the contribution of the α2β1 receptor to breast carcinoma function has been examined *in vitro*, as well as in *in vivo* model systems. Downregulation of α2 subunit expression in a well-differentiated breast carcinoma cell line using antisense oligonucleotides resulted in a transition from a contact-inhibited epithelioid morphology to a more mesenchymal, fibroblastic morphology indicative of a poorly differentiated carcinoma (31). Conversely, re-expression of α2 in a poorly differentiated, invasive breast carcinoma cell line caused the cells to regain their contact inhibition, form cell-cell contacts, and diminish their *in vitro* invasive potential (32). More importantly, the *in vivo* tumorigenicity of the α2-expressing cells was dramatically reduced (32). Taken together, these results support a role for α2β1 in regulating the differentiation of the breast epithelium and suppressing tumorigenicity and invasion. The signaling pathways that are activated by the α2β1 integrin to promote mammary epithelial differentiation have

not been identified. However, the information gained from these analyses should contribute greatly to our understanding of the early stages of breast carcinoma progression.

Interestingly, the mechanism by which the α2β1 integrin modulates differentiation may be related, in part, to the up-regulation of the α6 and β4 integrin subunits. The expression of these two subunits, but not other integrin subunits, was significantly enhanced when the α2 subunit was overexpressed in poorly differentiated breast carcinoma cells (33). When either the α6 or β4 subunit was overexpressed independently of the α2 subunit many, but not all, of the differentiated characteristics that were observed for the α2 transfectants were recapitulated (33). For example, the α6 and β4 transfectants became contact inhibited and their growth was diminished, but they did not form highly branched, duct-like structures when grown within a collagen gel. In addition, the α6 and β4 transfectants were more invasive *in vitro* than the parental cell line contrasting with decreased invasion observed for the α2 transfectants. This involvement of the α6β4 receptor in carcinoma invasion is discussed later.

THE α6β1 INTEGRIN

There are conflicting data in the literature with regard to the expression of the α6 subunit in breast carcinoma. As with the α2β1 receptor, the level and the pattern of expression of the α6 subunit in benign lesions such as fibrocystic disease and fibroadenomas are similar to those observed for the normal mammary gland (8). α6 expression is strongest in the myoepithelial cells and the staining is predominantly polarized at the basal surface in contact with the basement membrane. However, disagreements arise about the analysis of expression levels of the α6 subunit in adenocarcinomas of the breast. A loss or absence of α6 staining in poorly differentiated invasive ductal and lobular carcinomas has been described in several studies (34–36). In contrast, other reports suggest that α6 expression is retained in many breast adenocarcinomas and the localization of expression suggests an important role in malignancy (7,8,37). For example, consistent staining of the α6 subunit in pseudopodial-like extensions of invasive lobular carcinoma suggests a possible role for this integrin in the motile phenotype of these cells (7,8).

One difficulty in assessing the importance of integrin receptors to breast carcinoma progression based upon expression patterns alone is that the data are not usually correlated with patient outcome or survival. However, Imhof and colleagues reported that high expression of the α6 subunit in women with breast cancer correlated significantly with reduced survival times (37). In their analysis of 119 patients with invasive breast carcinoma, all of the patients with low or absent α6 expression survived while the mortality rate of the patients with a high level of α6 expression was 19%. These correlations with survival were stronger, in fact, than those observed with estrogen receptor status. Of note, 30 out of 34 of the patients who presented with distant metastases were highly positive for α6 expression. Taken together, these analyses provide strong support for a role for α6 containing integrin receptors in the promotion of metastatic breast cancer.

It must be remembered that the α6 subunit can associate with both the β1 and β4 subunits to form the α6β1 and α6β4 receptors (1). The results from the Imhof study do not distinguish between these two combinations leaving open the question of whether one or the other, or both, are responsible for promoting the metastatic potential of breast carcinoma. Recently, results of a similar study examining the correlation of β4 expression with survival rates of breast cancer patients were reported. Patients with tumors that expressed both the β4 subunit and specific laminins, ligands for both α6β1 and α6β4, had increased mortality rates (38). This study highlights an additional caveat in the use of expression data alone to interpret integrin function, i.e., if the appropriate extracellular matrix ligand is not present, integrin receptors may be expressed but not functional. Although the findings support the involvement of α6β4 in tumor progression, they do not negate a role for α6β1 because immunohistochemical analyses cannot distinguish relative levels of α6β1 and α6β4 on the cell surface. Fortunately, this important question is being addressed in several mechanistic studies and it appears that both the α6β1 and α6β4 integrins can play important roles in breast cancer progression.

To address the contribution of the α6β1 receptor to breast cancer, a dominant-negative technique for 'knocking-out' the expression of α6β1 in MDA-MB-435 cells, a human breast carcinoma cell line that is highly metastatic in athymic mice, was developed (39). A mutant β4 cDNA that lacked most of the cytoplasmic domain was transfected into the MDA-MB-435 cell line, which does not express the β4 subunit. The truncated β4 subunit acted as a dominant negative by associating with the endogenous α6 and depleting

functional α6β1 on the cell surface. Elimination of α6β1 expression inhibited the ability of these cells to mediate specific *in vitro* functions associated with metastatic spread such as laminin-1 adhesion and migration (39). When these cells were inoculated into the mammary fat pad of nude mice, primary tumor size was significantly diminished because of a combined decrease in proliferation and increase in apoptosis (40). More importantly, the α6β1 deficient cells could not survive as secondary metastases in the lungs or liver because they underwent apoptosis (40). Taken together, these data indicate that the α6β1 integrin contributes to breast carcinoma progression by promoting the growth and survival of breast carcinomas. It should be noted that the α6β1 receptor has also been implicated in the binding of breast carcimoma cells to the endothelium, a function that could also contribute to its promotion of metastasis (41).

The specific signaling pathways that are regulated by α6 to promote the growth and survival of mammary carcinoma cells could be important targets for inhibitors of metastasis. Although these pathways remain to be elucidated, a clue may be found in a recent report that described a role for the α6β1 receptor in the survival of normal mammary epithelial cells (42). These cells require both α6β1 ligation and insulin or insulin-like growth factor-1 (IGF-1)[3] to prevent apoptosis when they are grown *in vitro*. Ligation of α6β1 does not influence the phosphorylation of the growth factor receptors but it does increase the activation of downstream signaling molecules including the insulin receptor substate-1, the lipid kinase phosphoinositide 3-OH kinase (PI3K), and the serine/threonine kinase Akt/PKB. Activation of PI3K was essential for the survival effect. This finding adds to the growing number of studies that support the contribution of PI3K and Akt/PKB to the survival of many different cells (43–45). With regard to the survival of breast carcinoma cells, the discovery that α6β1 cooperates with IGF-1 to promote survival is intriguing because the IGF-1 receptor has been implicated in the survival of many different tumor cell types, including breast (46). It is not unlikely that a pathway involved in normal cell function would be utilized by transformed cells and this possibility warrants further investigation.

[3] Abbreviations: insulin-like growth factor-1 (IGF-1); phosphoinositide 3-OH kinase (PI3K); intracellular cAMP concentration ([cAMP]i); epidermal growth factor receptor (EGFR).

THE α6β4 INTEGRIN

The role of the α6β4 integrin in breast carcinoma progression is a complex one and represents the best example of how difficult it is to understand the contribution of an integrin receptor to carcinoma progression based upon expression data alone. In normal epithelial cells, the α6β4 integrin participates in the formation of hemidesmosomes, very stable, adhesive structures that are not conducive to cell motility (15,16,47,48). For this reason, it had long been thought that a loss of β4 expression would be necessary for the acquisition of the motile phenotype characteristic of invasive carcinomas. In fact, many of the early immunohistochemical reports supported this hypothesis because a decrease in β4 expression was detected (49). In contrast, as mentioned previously, more recent studies have demonstrated β4 expression in aggressive breast carcinomas (38), as well as many other types of carcinoma (49).

The mechanism by which the α6β4 integrin contributes to breast carcinoma progression has been addressed by *de novo* expression of the β4 subunit in cell lines that express α6β1 and not α6β4. When the β4 subunit was transfected into the MDA-MB-435 cell line, a threefold-fourfold increase in the *in vitro* invasive potential of these cells was observed (50). As mentioned previously, this increase in invasion was also observed when β4 was overexpressed in a murine mammary carcinoma cell line (33). Similar increases in invasion after α6β4 expression have been reported for a colon carcinoma cell line, RKO (51). The apparent paradox in α6β4 function in normal epithelia and carcinomas has been resolved by the recent demonstration that the α6β4 receptor can interact with the actin cytoskeleton in invasive carcinoma cells and that α6β4 contributes to the formation of motility structures such as filopodia and lamellae in these cells (52). The concensus from these data is that the α6β4 receptor contributes to carcinoma progression through its ability to promote motility and invasion.

The data implicating α6β4 in invasion support the role of this integrin as a tumor promoter. However, this conclusion is contradicted by the finding that expression of the β4 subunit in the RKO colon carcinoma cell line also resulted in an increase in cell death (53). These tumor-promoting and tumor-suppressing activities of α6β4 could conflict with one another and complicate the understanding of α6β4 function in carcinoma progression. Interestingly, an increased level of apoptosis was not observed in the MDA-MB-435/

β4-transfected cells (54). Further analysis of the differences between the RKO and MDA-MB-435 cells has revealed that they differ in their p53 status: RKO cells express wild type p53 whereas MDA-MB-435 cells express mutant p53 (54,55). After expression of a dominant negative p53 mutant in the RKO cells, the ability of α6β4 to enhance apoptosis was abolished (54). These exciting findings underscore the fact that integrin expression alone cannot be used to assess function because the regulation and signaling capabilities of these receptors are influenced by many other factors.

Based on our knowledge to date, a model for α6β4 function in breast carcinoma progression can be proposed. An initial decrease in α6β4 expression, or altered α6β4 function, may be required for the early stages of tumor development if p53 is still functional. In the absence of p53 activity, re-expression or activation of α6β4 may promote carcinoma progression by enhancing the invasive phenotype and metastasis. One intriguing study in which a primary tumor that was α6β4 deficient and an autologous lymph node lesion that was α6β4 positive were both identified is consistent with this model (35).

Given a role for α6β4 in carcinoma invasion, it is imperative to determine the mechanism involved so that methods for intervention can be developed. Investigation of the signaling pathways involved in the α6β4-dependent promotion of invasion revealed that PI3K activity was required for invasion (50). In keeping with this requirement, α6β4 activated PI3K to a greater extent than α6β1 or other β1 integrins (50). The importance of PI3K to carcinoma invasion was confirmed by the ability of a constitutively active PI3K to promote MDA-MB-435 invasion in the absence of α6β4 expression, and conversely, a dominant negative PI3K p85 subunit to inhibit α6β4-dependent invasion (50). The involvement of PI3K in breast carcinoma invasion was also demonstrated in an independent study in which the invasive potential of T47D breast carcinoma cells was diminished by inhibitors of PI3K signaling (56). Taken together, the data implicating the activity of PI3K in both α6β1 and α6β4-dependent functions support a central role for this kinase and its lipid products in breast carcinoma progression and highlight the need to investigate this signaling pathway in more detail for its therapeutic potential.

In addition to PI3K itself, downstream effectors that mediate the action of PI3K could also be targets for therapy. One such molecule that has been shown to be essential for PI3K-dependent invasion is the small G-protein Rac (50). Rac belongs to the Rho small G-protein family, the members of which participate in modification of the actin cytoskeleton in response to many stimuli (57). In fibroblasts, expression of constitutively active Rac results in the formation of membrane ruffles and lamellipodia, structures that are indicative of a motile phenotype (57). In carcinoma cells, lamellae formation is also dependent upon PI3K activity and Rac is essential for this function (50). However, Rac alone is not sufficient to promote the invasion of MDA-MB-435 cells indicating that other downstream effectors of α6β4 and PI3K are required. In contrast, expression of constitutively active Rac, and also Cdc42, increased the invasive potential of T47D breast carcinoma cells in the absence of other stimuli (56). Although many factors could differ between these two carcinoma cell lines, a plausible explanation for this discrepancy could be related to their level of differentiation. MDA-MB-435 cells are poorly differentiated and they do not express E-cadherin or form cell-cell interactions (58). In contrast, T47D cells are more well differentiated and maintain cell-cell adhesion through E-cadherin interactions (58). In normal epithelial cells, E-cadherin-mediated cell-cell interactions can be modified by the action of Rho family members on the actin cytoskeleton (59). Therefore, given the large amount of data correlating the loss of E-cadherin with increased cell motility (60,61), this could be the mechanism by which Rac and Cdc42 increase the invasion of T47D cells. Nevertheless, in both systems Rac plays an important role in the invasive phenotype and it should be considered an important candidate for the development of inhibitors of invasion.

Akt/PKB is a another downstream effector of PI3K that is a potential target for the inhibition of carcinoma progression. In the absense of Akt/PKB function, cells undergo apoptosis upon growth factor withdrawal or, in the case of epithelial cells, upon detachment from the ECM (43–45). Akt/PKB function is also required for the survival of transformed cells (43). As described earlier, Akt/PKB activation may play a role in the α6β1-dependent survival of breast carcinoma cells and, therefore, inhibition of this PI3K effector would decrease their ability to metastasize. Of note, Akt/PKB function is not required for carcinoma invasion even though it is activated by α6β4 (50). This observation suggests that the PI3K effectors that are involved in invasion are distinct from those involved in survival. Given that carcinoma progression involves both tumor cell invasion and survival, disruption of either the Rac or Akt/PKB pathways should limit the development of metastases.

The ability of α6β4 to promote invasion does not depend on PI3K activation alone but involves other signaling pathways as well. The recent demonstration that α6β4 stimulates the chemotactic migration and invasion of breast carcinoma cells by suppressing the intracellular cAMP concentration ($[cAMP]_i$) through the activation of a cAMP-specific phosphodiesterase (62) provides an additional target for intervention. The decreased $[cAMP]_i$ allows the propagation of chemotactic signals that would otherwise be inhibited, or "gated", at higher $[cAMP]_i$ Although there have been reports of cAMP regulating PI3K activity and both pathways are involved in lamellae formation, the data suggest that α6β4 regulates PI3K and the phosphodiesterase through distinct mechanisms and that they function independently of one another (50,62).

What is the mechanism by which α6β4 activates the signaling pathways that are important for carcinoma progression? The β4 subunit has an extremely long cytoplasmic domain (1000 amino acids) compared to other integrin β subunits (30–50 amino acids) and it is required for the signaling functions of α6β4 (12–14,50). Surprisingly, very little is known about the interactions of this large cytoplasmic domain as they relate to its signaling properties and the few studies that have examined the signaling functions of α6β4 have not been definitive. For example, the β4 cytoplasmic domain is phosphorylated on tyrosine, as well as serine and threonine residues upon clustering or ligation of the α6β4 receptor (63). However, the mechanism for this phosphorylation is unknown and the specific residues that are modified have not been identified. Shc has been reported to be phosphorylated and to associate with the β4 cytoplasmic domain upon ligation but its specific binding site in β4 has not been identified (63). In addition, a direct binding motif for the p85 regulatory subunit of PI3K is not present in the β4 cytoplasmic domain, suggesting that this lipid kinase is activated through intermediate signaling molecules (64). It is clear that a more detailed and rigorous analysis of the β4 subunit is needed to understand how α6β4 signals invasion. Only then will it be possible to devise methods to interfere with the activation of its specific signaling pathways that contribute to carcinoma progression.

INTEGRIN COOPERATIVITY WITH GROWTH FACTORS

An emerging area in the study of integrin contributions to cancer is the cross-talk between these adhesion receptors and soluble growth factor and cytokine receptors (4). As mentioned previously, in the normal breast integrin receptors cooperate with hormones and growth factors to promote mammary epithelial differentiation and function (18). An increasing number of studies indicate that integrins also cooperate with soluble factors to promote carcinoma progression. From these studies, several mechanisms for cooperation have been revealed that may be useful in the future manipulation of these pathways to inhibit progression.

Integrins can cooperate with growth factor receptors to enhance their signaling capabilities (4; Fig. 1A). An example of this, as mentioned previously, is the cooperation of the α6β1 integrin with the IGF-1 receptor to activate cell survival pathways in normal mammary epithelial cells (42). Integrin receptors can also associate directly with growth factor receptors to enhance signaling. One example that is especially relevant for breast cancer is the finding that the α6β1 and α6β4 integrins can associate with the ErbB2 protein and increase its signaling functions (65). This cooperation between α6β4 and ErbB2 could also be a mechanism through which α6β4 activates its own downstream signaling pathways. ErbB2 is a member of the epidermal growth factor receptor (EGFR) family and its overexpression in breast cancer correlates with a poor prognosis (66). *In vitro*, antibodies specific for ErbB2 inhibit the growth of ErbB2 overexpressing breast carcinoma cells. The recent success of a recombinant, humanized ErbB2 antibody (Herceptin) in clinical trials confirms the relevance of the ErbB2 receptor as a therapeutic target (67). It is intriguing, therefore, to consider the possibility that methods to disrupt the interaction between α6β4 and ErbB2, and interfere with signaling functions, could also have therapeutic potential.

Another mechanism of integrin-growth factor receptor cooperation involves integrin activation by growth factors and cytokines (Fig. 1B). A well studied example is the activation of the αvβ5 integrin by the insulin-like growth factor receptor (68,69). Breast carcinoma cells that express αvβ5 adhere to but do not migrate on vitronectin *in vitro* unless they are stimulated with IGF-1 or insulin. *In vivo*, these cells form tumors in the absence of IGF-1, however, they only metastasize when stimulated with IGF-1 or insulin (68). These data suggest that the cooperation between αvβ5 and the IGF-1 receptor may regulate migration and invasion to promote the metastatic spread of tumor cells.

A final intriguing mechanism for cooperation involves cross-talk between integrin and growth factor

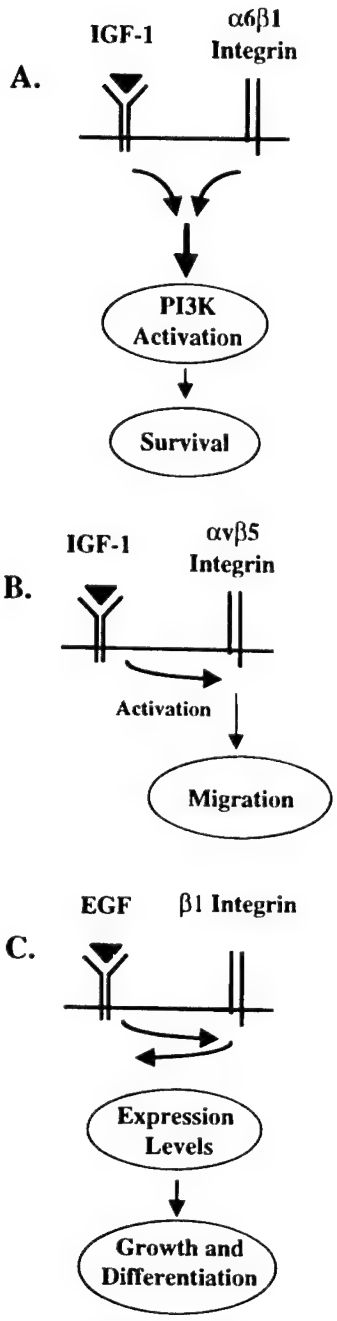

Fig. 1. Schematic representation of cooperativity between growth factor and integrin receptors. (A). Growth factor receptor and integrin-dependent signals are amplified when both receptors are engaged. (B). Integrin function is activated in response to growth factor receptor signaling. (C). Cross-talk between growth factor and integrin receptors can regulate the expression levels of each receptor.

receptors regulating the expression levels of these receptors (Fig. 1C). The growth and morphology of a breast carcinoma cell line cultured within a 3-D basement membrane matrix could be altered by inhibiting the function of either the β1 integrin subunit or the EGFR resulting in a concommitant decrease in the expression of the other (70). The reciprocal modulation of the expression of these receptors was controlled through MAPK (70). This cross talk confirms the notion that the normal differentiation of the mammary epithelium is controlled by a tightly regulated balance of signaling pathways and that disruption of this balance can contribute to breast carcinoma progression.

CONCLUSIONS

The purpose of this review has been to examine the contribution of integrin receptors to breast carcinoma progression and to highlight some of the possible targets of integrin signaling pathways for therapeutic development. It is clear from this analysis that specific integrins play an important role in breast cancer and that they have the potential to be manipulated for intervention in tumor progression. Several key areas emerge that deserve further examination. The cross-talk between receptors for soluble factors (growth factors, hormones, and cytokines) and integrin receptors is an exciting area that has just begun to be appreciated. Most likely, more examples of cooperativity will emerge and the challenge will be to decipher the mechanisms involved as well as the specific contributions of each to cancer progression. With regard to integrin-regulated signaling pathways, there is solid evidence to support a pivotal role for PI3K in breast carcinoma progression. The activation of this lipid kinase is required for two essential functions of progression, invasion and survival. Understanding how specific integrin receptors activate this signaling pathway, either independently or in cooperation with growth factors, will be an important focus in future studies. Finally, more detailed and mechanistic analyses of the contributions of integrin receptors to breast carcinoma progression are needed. Although strong correlations between integrins and breast cancer have been established, more work remains to unravel the specific mechanisms that are responsible for the effects of these integrins on tumor growth, progression and metastasis.

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Activation of Phosphoinositide 3-OH Kinase by the α6β4 Integrin Promotes Carcinoma Invasion

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Summary

We demonstrate that the α6β4 integrin promotes carcinoma invasion through a preferential and localized targeting of phosphoinositide-3 OH kinase (PI3K) activity. Stable expression of α6β4 increased carcinoma invasion in a PI3K-dependent manner, and transient expression of a constitutively active PI3K increased invasion in the absence of α6β4. Ligation of α6β4 stimulated significantly more PI3K activity than ligation of β1 integrins, establishing specificity among integrins for PI3K activation. α6β4-regulated PI3K activity was required for the formation of lamellae, dynamic sites of motility, in carcinoma cells. The small G protein Rac is required downstream of PI3K for invasion. These studies define a mechanism by which the α6β4 integrin promotes carcinoma invasion and invoke a novel function for PI3K signaling.

Introduction

Understanding the progression from carcinoma in situ to invasive carcinoma is one of the most complex and challenging problems in the pathobiology of cancer. Because the biology of invasive carcinoma is essentially an aberration of epithelial cell biology, insight into this problem has been obtained by comparing the properties of epithelial and carcinoma cells. Distinguishing features of epithelia are their polarized morphology, attachment to an underlying basement membrane, and presence of specialized cell–cell contacts (Rodriguez-Boulan and Nelson, 1989). The progression to invasive carcinoma has been shown to involve perturbations in these features resulting in the acquisition of a motile, mesenchymal phenotype. This progression involves alterations in the expression and function of surface receptors that maintain the epithelial phenotype. Most notably, invasive carcinoma is characterized by a loss of function of cadherins, cell–cell adhesion receptors (Takeichi, 1993; Birchmeier et al., 1995). Integrins, in contrast, are essential for both normal epithelial function and for mediating dynamic processes associated with invasive carcinoma such as migration (Hynes, 1992). For this reason, it is assumed that the altered expression and function of specific integrins contribute significantly to invasive carcinoma (Juliano and Varner, 1993). Although considerable progress has been made in understanding how integrins regulate cell function, relatively little is known about integrin specificity and integrin-mediated signaling events in the pathogenesis of invasive carcinoma.

The integrin α6β4, a receptor for the laminins, is likely to play a pivotal role in the biology of invasive carcinoma (Rabinovitz and Mercurio, 1996). This integrin is essential for the organization and maintenance of epithelial structure (Dowling et al., 1996; Vanderneut et al., 1996). In many epithelia, this integrin mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with the extracellular matrix (Borradori and Sonnenberg, 1996; Green and Jones, 1996). Expression of the α6β4 integrin persists, however, in many tumor cells that do not form stable adhesive contacts but rather exhibit the motile phenotype characteristic of invasive carcinoma. Indeed, numerous pathological studies have correlated α6β4 expression and localization with invasive carcinoma (Falcioni et al., 1986; Van Waes et al., 1991; Serini et al., 1996). Notable examples of the association between α6β4 expression and carcinoma include the finding that this integrin is not expressed in normal thyroid, but induction of its expression correlates with the progression to invasive thyroid carcinoma (Serini et al., 1996). Also, expression of the α6β4 integrin is enhanced at the invading fronts of gastric carcinomas (Tani et al., 1996). Such correlative studies have been substantiated by our finding that expression of α6β4 in a β4-deficient colon carcinoma cell line dramatically increases the invasive potential of these cells (Chao et al., 1996). Also, we demonstrated recently that α6β4 mediates the migration of invasive colon carcinoma cells on laminin-1 through its ability to associate with the actin cytoskeleton and promote the formation and stabilization of filopodia and lamellae (Rabinovitz and Mercurio, 1997). This finding implies that the function and cytoskeletal association of α6β4 in invasive carcinoma cells are distinct from its established role of anchoring epithelial cells to the basement membrane through its association with cytokeratins. Together, the current data indicate a key role for the α6β4 integrin in promoting carcinoma invasion, and they suggest that understanding the signaling pathways regulated by this integrin should provide insight into the mechanism of invasion.

Much of the work on signaling through the α6β4 integrin to date has focused on the structurally distinct, 1000 amino acid cytoplasmic domain of the β4 subunit (Hemler et al., 1989; Kajiji et al., 1989; Kennel et al., 1989). In keratinocytes, the β4 cytoplasmic domain has been shown to bind the adaptor protein Shc and activate the Ras-Mitogen activated protein kinase (MAPK) pathway, a pathway implicated in α6β4-mediated regulation of keratinocyte proliferation (Mainiero et al., 1997). The argument could be made that activation of MAPK by α6β4 is relevant for invasion because of the report linking MAPK to cell migration through its ability to phosphorylate myosin light chain kinase (Klemke et al., 1997).

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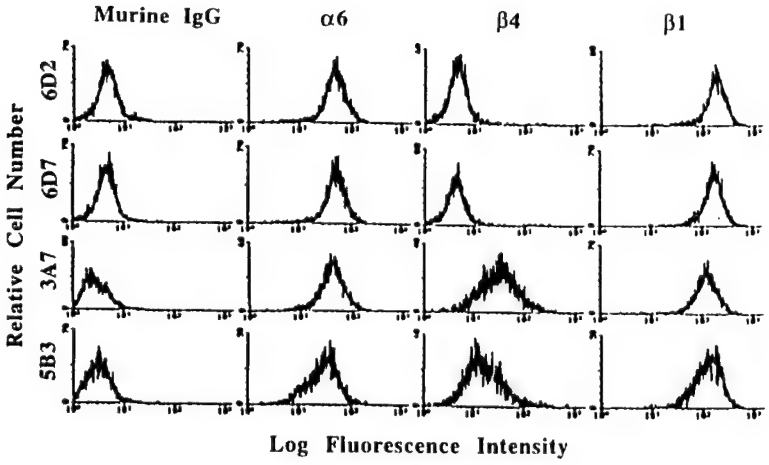

Figure 1. Surface Expression of Integrin Subunits in MDA-MB-435 Transfectants

Subclones of transfected MDA-MB-435 cells expressing β4 on the cell surface were isolated by FACS using UM-A9, a MAb specific for the β4 integrin subunit. MDA-MB-435 cells transfected with vector alone (6D2 and 6D7) or the human β4 integrin subunit (3A7 and 5B3) were analyzed by flow cytometry using monoclonal antibodies specific for the indicated integrin subunits.

No study, however, has addressed the possibility that MAPK or other signaling molecules downstream of α6β4 contribute to carcinoma invasion and metastasis. Indeed, the possibility exists that the signaling pathways required for invasion differ from those involved in cell proliferation. In addition, functions of α6β4 that contribute to invasion may also differ either qualitatively or quantitatively from signaling pathways regulated by other integrins, especially given the size and structural diversity of the β4 cytoplasmic domain.

In this study, we sought to identify signaling pathways through which the α6β4 integrin promotes invasion. For this purpose, we used a model system in which expression of this integrin in α6β4-deficient breast carcinoma cells markedly increases their rate of invasion. We also used a colon carcinoma model in which the α6β4 integrin is known to mediate motile events required for invasion. The data obtained reveal that PI3K (phosphoinositide-3 OH kinase) and the downstream effector Rac are required for carcinoma invasion. We also demonstrate that α6β4 activates PI3K preferentially over α6β1 and other β1 integrins and that this α6β4-regulated PI3K activity is required for the formation of lamellae, dynamic sites of motility in invasive carcinoma cells.

Results

Expression of the α6β4 Integrin Increases the Invasiveness of MDA-MB-435 Cells

The MDA-MB-435 cells used in this study do not express the α6β4 integrin, although they express the α6β1 integrin (Shaw et al., 1996). Stable subclones of these cells were generated that express either the α6β4 integrin or a mutated α6β4 that lacks the β4 cytoplasmic domain with the exception of the four amino acids proximal to the transmembrane domain (β4-ΔCYT). The relative surface expression of the α6, β4, and β1 subunits on the subclones used in this study is shown in Figure 1. Expression of the β4 subunit did not alter surface expression of the α6 subunit (Figure 1) or other integrin α subunits (data not shown) on these cells. However, a slight decrease in β1 surface expression was observed in the β4 transfectants that probably reflects a decrease in α6β1 expression at the expense of α6β4 expression (Figure 1).

The possibility that expression of the α6β4 integrin stimulates the invasion of carcinoma cells was examined by comparing the ability of mock transfectants (6D2 and 6D7) and β4 transfectants (3A7 and 5B3) to invade Matrigel in a standard chemoinvasion assay (Albini et al., 1987). As shown in Figure 2A, the rate of invasion of the β4 transfectants was approximately 3- to 4-fold greater than that of the mock transfectants in a 4 hr assay. The β4-ΔCYT transfectants invaded at a slightly slower rate than that of the mock transfectants (Figure 2A), indicating that the β4 cytoplasmic domain is essential for stimulating invasion. Interestingly, the rate of adhesion to laminin was not greater in the β4 transfectants than in the mock transfectants (data not shown).

Antibodies Specific for the α6β4 Integrin Stimulate Invasion of MDA-MB-435 Cells

To examine the contribution of integrin receptors to the invasion of MDA-MB-435 cells, Matrigel chemoinvasion assays were performed in the presence of integrin subunit-specific antibodies. A β1-specific antibody (MAb 13) inhibited invasion of the mock and β4 transfectants (Figure 2B). An α6-specific MAb (2B7) inhibited invasion of the mock transfectants by approximately 60% (Figure 2B), in agreement with our previous result that these cells use α6β1 as a major laminin receptor (Shaw et al., 1996). However, the same antibody increased the rate of invasion of the β4 transfectants by approximately 30% (Figure 2B). The stimulation of invasion observed for the α6 antibody in the MDA-MB-435/β4 transfectants suggests that α6β4 is not required for the adhesive functions involved in invasion but rather acts as a signaling receptor whose function can be enhanced by antibody binding. Such a phenomenon has been observed for stimulation of melanoma invasion by αv-specific antibodies (Seftor et al., 1992). This possibility is also supported by the finding mentioned above that α6β4 expression did not increase the rate of adhesion of MDA-MB-435 cells to laminin-1. These data indicate that the adhesive functions of the MDA-MB-435/β4 transfectants required for invasion are mediated largely by β1 integrins.

Invasion of MDA-MB-435 Cells Is Dependent on PI3K

As a prelude to identifying the signaling mechanism by which the α6β4 integrin stimulates invasion, we assessed first the effects of the MAPK kinase inhibitor

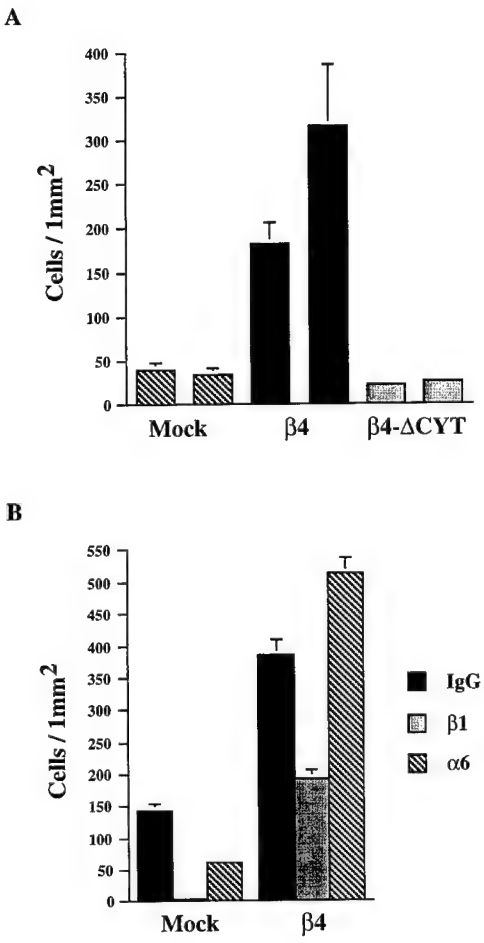

Figure 2. Invasion of the MDA-MB-435 Transfectants

(A) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel. Matrigel was diluted in cold distilled water, added to the upper well of Transwell chambers, and dried in a sterile hood. The Matrigel was reconstituted with medium and the transfectants (5×10^4) were added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers.

(B) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel in the presence of monoclonal antibodies specific for the indicated integrin subunits. Cells were preincubated for 30 min in the presence of antibodies before addition to the Matrigel-coated wells. After 4 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Experimental Procedures.

Mock, MDA-MB-435 cells transfected with vector alone; β4, MDA-MB-435 cells transfected with the full-length β4 subunit; β4-ΔCYT, MDA-MB-435 cells transfected with the β4 subunit lacking the cytoplasmic domain; IgG, nonspecific antibody; β1, MAb 13; α6, 2B7. The data shown are from (A) two individual subclones of each transfectant and are the mean values (±SD) of a representative experiment done in duplicate, (B) the mean values (±SEM) of a representative experiment done in triplicate.

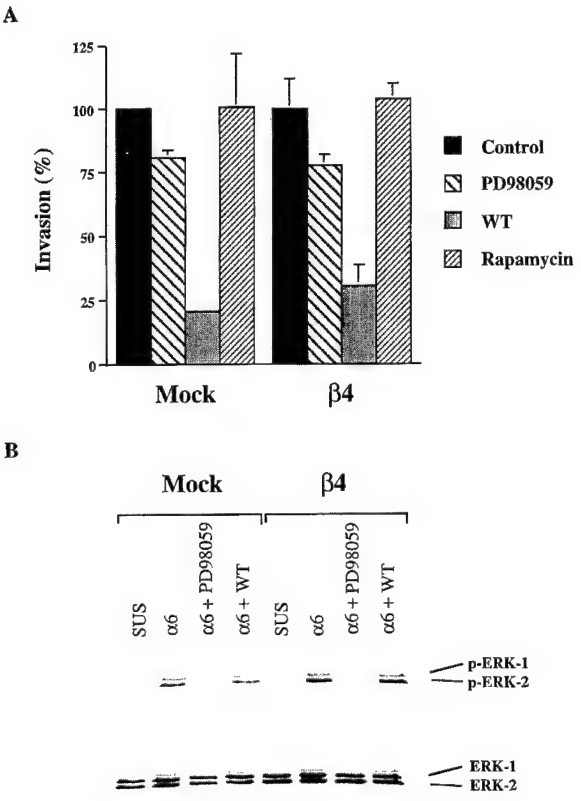

Figure 3. Analysis of MAPK, PI3K, and p70 S6K Involvement in MDA-MB-435 Invasion

(A) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel in the presence of either the MEK inhibitor PD98059 (25 μM), the PI3K inhibitor wortmannin (100 nM), or the p70 S6K inhibitor rapamycin (20 ng/ml). Cells were preincubated for 10 min in the presence of the inhibitors before addition to the Matrigel-coated wells. After 4 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Experimental Procedures. The data shown are the mean values (±SD) of a representative experiment done in duplicate.

(B) MDA-MB-435 transfectants were maintained in suspension or incubated with α6-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 min in the absence or presence of the indicated inhibitors. Aliquots of total cell extracts were normalized for protein content and resolved by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and immunoblotted using an ERK polyclonal antibody that recognizes the phosphorylated isoforms of ERK-1 and ERK-2 (upper panel). The blots were then stripped and reprobed with an ERK-1 antibody that recognizes both ERK-1 and ERK-2 (lower panel).

Mock, MDA-MB-435 cells transfected with vector alone; β4, MDA-MB-435 cells transfected with the full length β4 subunit; WT, wortmannin; SUS, cells maintained in suspension.

PD98059 on MDA-MB-435 invasion (Dudley et al., 1995; Pang et al., 1995). As shown in Figure 3A, pretreatment of these cells with PD98059 (25 μM) resulted in only a modest inhibition (20%) of invasion. To confirm that PD98059 inhibits MAPK activity in these cells, an antibody that recognizes the phosphorylated, active isoforms of ERK1 and ERK2 was used. Antibody-induced clustering of the α6 integrins in both the mock and β4 transfectants using 2B7, an α6-specific Ab, stimulated activation of MAPK as assessed by reactivity with the phospho-specific MAPK Ab (Figure 3B). This activation was inhibited by PD98059 (25 μM). Of note, α6β4 expression did not have a significant impact on α6-induced MAPK activation in these cells.

We next targeted PI3K as a mediator of invasion because of its central involvement in multiple signaling pathways (Toker and Cantley, 1997). The PI3K inhibitor wortmannin (WT) (Ui et al., 1995) inhibited invasion of

both the mock and β4 transfectants by 70%–80% (Figure 3A). In contrast to PD98059, WT did not inhibit activation of MAPK by antibody-induced clustering of the α6 integrins in either the mock or β4 transfectants (Figure 3B). Taken together, these results suggest that PI3K, but not MAPK, is necessary for the invasion of MDA-MB-435 cells. The total amount of ERK-1 and ERK-2 protein was not altered by either PD98059 or WT (Figure 3B).

Activation of PI3K by the α6β4 Integrin

To determine if the α6β4 integrin can stimulate PI3K activity, in vitro kinase assays were performed using the mock, β4, and β4-ΔCYT transfectants of MDA-MB-435 cells. After ligation of the α6 integrins with 2B7, extracts were immunoprecipitated with a phosphotyrosine-specific antibody to capture the activated population of PI3K, and these immunoprecipitates were assayed for their ability to phosphorylate crude brain phosphoinositides. As shown in Figure 4A, an increase in PI3K activity, indicated by the appearance of PtdIns-3,4,5-P_3, was observed upon clustering the α6β1 integrin in the mock transfectants and the α6β4 integrin in the β4 transfectants. More importantly, PI3K activity stimulated by clustering the α6β4 integrin was markedly greater than that observed after clustering the α6β1 receptor. This enhanced stimulation of PI3K was also seen using a β4-specific MAb to ligate the α6β4 integrin in the β4 transfectants (Figure 4B).

PI3K activity was higher in the β4 transfectants than in the mock transfectants after adhesion to laminin-1 (Figure 4A). This observation suggests that interactions with laminin through this receptor can stimulate PI3K activity even though α6β4 is not used as an adhesion receptor in these cells. PI3K activity was not increased upon ligation of the α6β4-ΔCYT receptor, and little activity was evident when the transfectants were maintained in suspension (Figure 4).

Our data suggested that the ability of the α6β4 integrin to activate PI3K may be quantitatively greater than that of β1 integrins in MDA-MB-435 cells. This possibility was examined by comparing PI3K activation in the β4 transfectants in response to antibody ligation of either β1 integrins or the α6β4-integrin. As shown in Figure 4B, ligation of the α6β4 integrin with β4-specific antibodies stimulated PI3K activity approximately 2-fold greater than β1 integrin ligation, demonstrating that PI3K is activated preferentially by the α6β4 integrin. The differences between the abilities of the α6β4 and β1 integrins to activate PI3K are most likely even greater than what was observed given the 2- to 3-fold higher level of expression of β1 than β4 integrins on the cell surface (Figure 1).

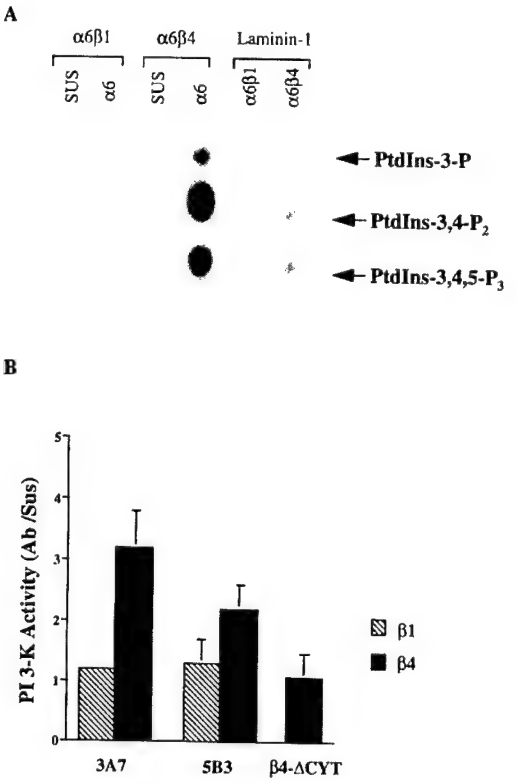

Figure 4. Analysis of PI3K Activity in the MDA-MB-435 Transfectants

(A) MDA-MB-435 transfectants were maintained in suspension or incubated with integrin-specific antibodies and allowed to adhere to anti-mouse IgG coated plates or laminin-1 coated plates for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine MAb 4G10 and Protein A sepharose for 3 hr. After washing, the beads were resuspended in kinase buffer and incubated for 10 min at room temperature. The phosphorylated lipids were resolved by thin layer chromatography. The D3-phosphoinositides are indicated by arrows. SUS, cells maintained in suspension; α6, cells clustered with the α6-specific antibody.

(B) The amount of radiolabeled PtdIns-3,4,5-P_3 was determined for each condition by densitometry. The integrin-activated levels of PtdIns-3,4,5-P_3 were compared to the level observed for the cells that were maintained in suspension. The value from this ratio was determined to be the relative PI3K activity stimulated by each integrin subunit. The data shown are the mean values (±SD) from two representative experiments. 3A7, subclone of MDA-MB-435 cells transfected with the full-length β4 subunit; 5B3, subclone of MDA-MB-435 cells transfected with the full-length β4 subunit; β4-ΔCYT, MDA-MB-435 cells transfected with the β4 subunit lacking the cytoplasmic domain; β1, cells clustered with the β1-specific antibody (MAb 13); β4, cells clustered with the β4-specific antibody (A9).

Constitutively Active PI3K Stimulates Invasion of MDA-MB-435 Cells

The hypothesis that the α6β4 integrin promotes invasion of MDA-MB-435 cells by enhancing the activity of PI3K implies that expression of a constitutively active form of PI3K in the parental cells should increase their invasion in the absence of α6β4 expression. To validate this prediction, a constitutively active, membrane-targeted PI3K (Myr-p110-Myc) was expressed transiently in the parental MDA-MB-435 cells, and the ability of these cells to invade Matrigel was compared to cells transfected with an empty vector. As shown in Figure 5A, constitutively active PI3K increased invasion 2-fold, and this invasion was inhibited by wortmannin. Expression of the transiently expressed p110 subunit was confirmed by immunoblotting using a myc-specific antibody (Figure 5A).

α6β4-Dependent Invasion Requires PI3K Activity

If the α6β4 integrin promotes invasion of MDA-MB-435 cells by enhancing the activity of PI3K, expression of a

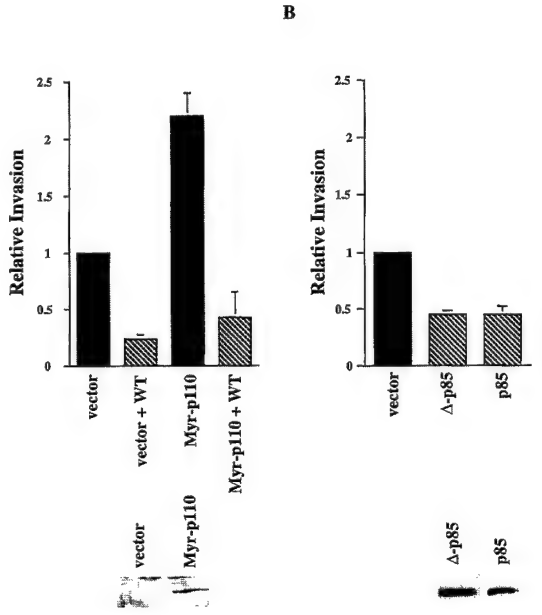

Figure 5. Analysis of PI3K Involvement in Invasion of MDA-MB-435 Cells by Transient Transfections

(A) MDA-MB-435 cells were transiently transfected with 1 μg pCS2-(n)β-gal and 4 μg of either the vector alone or a Myc-tagged, constitutively active form of the PI3K p110 catalytic subunit (Myr-p110) and assayed for their ability to invade Matrigel in the absence or presence of wortmannin (100 nM). The data shown are the mean values (±SD) of two (wortmannin) or three (without wortmannin) experiments done in triplicate.

(B) MDA-MB-435/β4 transfectants were transiently transfected with 1 μg pCS2-(n)β-gal and either the vector alone, 6 μg of a PI3K p85 subunit deleted in the p110 binding site (Δp85), or 6 μg of a wild-type PI3K p85 regulatory subunit (p85) and assayed for their ability to invade Matrigel. After 5 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained as described in Experimental Procedures. Invasion was quantitated by counting the cells that stained positively for β-galactosidase expression. Relative invasion was determined by comparing the amount of invasion obtained for the experimental transfections to that observed for the cells transfected with the vector alone, which was given the value of 1. The expression of the transfected cDNAs was confirmed by immunoblotting and is shown below each graph. The data shown are the mean values (±SD) of two experiments done in triplicate.

dominant negative PI3K subunit in the β4 transfectants should decrease their invasion. Transient expression of a GST-tagged, PI3K p85 subunit deleted in the p110 binding site (Δp85) inhibited invasion of the MDA-MB-435/β4 transfectants significantly (Figure 5B). A similar inhibition of invasion was observed after transient expression of a wild-type p85 subunit. It has been shown that overexpression of the wild-type p85 subunit blocks PI3K activation by binding to phosphotyrosine-containing proteins and inhibiting the binding of endogenous p85/p110 to these proteins (Rameh et al., 1995). The data obtained with the Δp85 and wild-type p85 subunits substantiate the wortmannin data shown in Figure 3A and confirm the involvement of PI3K signaling in α6β4-dependent invasion. Expression of the transiently expressed Δp85 and p85 subunits was confirmed by immunoblotting using GST- (Δp85) or myc-specific (wt p85) antibodies (Figure 5B).

The Akt/PKB Kinase and p70 S6 Kinase, Downstream Effectors of PI3K, Are Not Required for Invasion

The Akt/PKB serine/threonine kinase (Akt) and the p70 S6 kinase (S6K) are activated downstream of PI3K and, for this reason, could play important roles in invasion (Chou and Blenis, 1995; Franke et al., 1997). This possibility was supported by our finding that ligation of the α6β4 integrin in the MDA-MB-435/β4 transfectants activated both Akt and S6K (Figure 6A). Based on these observations, we examined the ability of parental MDA-MB-435 cells that expressed a constitutively active form of Akt (Myr-Akt) to invade Matrigel. Surprisingly, this constitutively active form of Akt actually decreased the rate of invasion in comparison to the control cells even though it was expressed at relatively high levels (Figure 6B). Most likely, the exogenously expressed active Akt sequestered a significant fraction of D3 phosphoinositides and precluded the use of these lipids in those signaling pathways downstream of PI3K that are required for invasion. We examined the involvement of S6K in invasion using rapamycin, a specific inhibitor of S6K activation (Chung et al., 1992; Kuo et al., 1992; Price et al., 1992). As shown in Figure 3, rapamycin did not decrease the invasion of either the mock or β4 transfectants. Based on these results, we conclude that Akt and S6K are not required for MDA-MB-435 invasion.

The Small G Protein Rac Is Required for MDA-MB-435 Invasion

The Rho family of small G proteins are involved in the actin rearrangements that result in the formation of stress fibers, membrane ruffles and lamellae, and filipodia (Nobes and Hall, 1995). The ability of cells to form these actin-containing structures is linked to their motility and therefore could influence their invasive potential (Sheetz, 1994; Rabinovitz and Mercurio, 1997). To examine this possibility, constitutively active mutants of either Rho (V14Rho), Rac (V12Rac), or Cdc42 (V12Cdc42) were transiently expressed in the parental MDA-MB-435 cells. As shown in Figure 6B, independent expression of these constitutively active small G proteins did not significantly alter the invasion of MDA-MB-435 cells, indicating that they are not sufficient by themselves to increase invasion.

To determine if either Rac or Cdc42 contributed to α6β4-dependent invasion, dominant negative mutants of Rac (N17Rac) and Cdc42 (N17Cdc42) were expressed in the MDA-MB-435/β4 transfectants. A significant reduction (50%) in invasion was observed when N17Rac was transiently expressed in the MDA-MB-435/β4 transfectants. In contrast, expression of N17Cdc42 did not inhibit invasion significantly (Figure 6C). To examine the role of Rac in PI3K-stimulated invasion of parental MDA-MB-435 cells, N17Rac was transiently expressed along with the Myr-p110-Myc construct. As shown in Figure 6D, coexpression of N17Rac inhibited the increased invasion that was observed when the constitutively active p110 subunit of PI3K was expressed alone. Taken together, these results demonstrate that Rac is an essential downstream mediator of the α6β4/PI3K signaling pathway involved in invasion. The inability of the constitutively active mutant of Rac to significantly increase

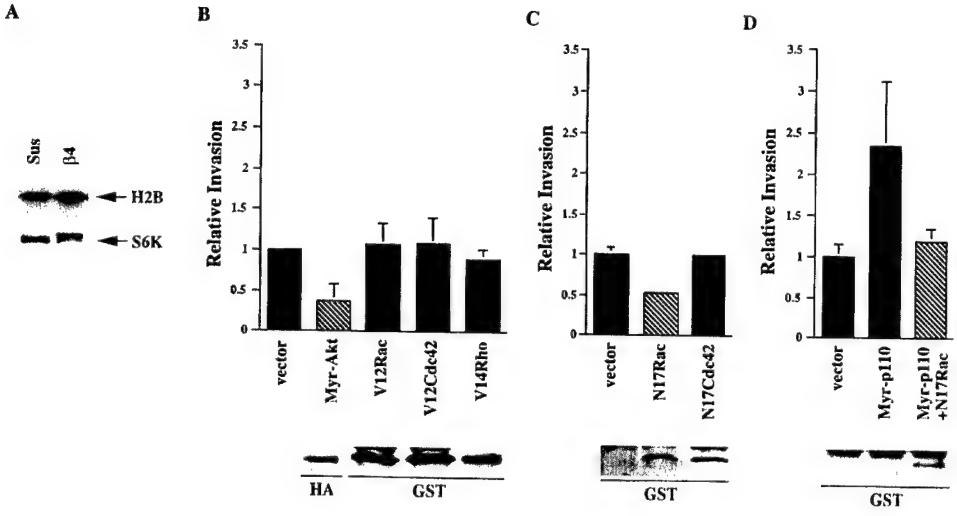

Figure 6. Analysis of Downstream Effectors in PI3K-Dependent Invasion of MDA-MB-435 Cells

(A) MDA-MB-435/β4 transfectants were maintained in suspension or incubated with α6-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 min. (Upper panel) Aliquots of cell extracts that contained equivalent amounts of protein were incubated with a polyclonal anti-Akt antibody and a 1:1 mixture of ProteinA/Protein G. After washing, the beads were resuspended in kinase buffer and incubated for 20 min at room temperature. The phosphorylated substrate, histone H2B, is indicated. (Lower panel) Aliquots of total cell extracts were normalized for protein content and resolved by 8% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and immunoblotted using a polyclonal antibody that recognizes the C-terminal end of p70 S6K.

(B) MDA-MB-435 cells were transiently transfected with 1 μg of pCS2-(n)β-gal and either 4 μg of the vector alone or 4 μg of constitutively active mutants of Akt (Myr-Akt), Rac (V12Rac), Cdc42 (V12Cdc42), or Rho (V14Rho) and assayed for their ability to invade Matrigel. After 5 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed and stained as described in Experimental Procedures. Invasion was quantitated by counting the cells that stained positive for β-galactosidase expression. Relative invasion was detemined by comparing the amount of invasion obtained for the experimental transfections to that observed for the cells transfected with the vector alone, which was given the value of 1. The data shown are the mean values (±SD) of 3 experiments done in triplicate.

(C) MDA-MB-435/β4 transfectants were transiently transfected with 1 μg of pCS2-(n)β-gal and either 4 μg of vector alone or 4 μg of dominant negative mutants of Rac (GST-N17Rac) or Cdc42 (N17Cdc42) and assayed for their ability to invade Matrigel as described above. The data shown are the mean values (±SD) of a representative experiment done in triplicate.

(D) MDA-MB-435 cells were transiently transfected with 1 μg of pCS2-(n)β-gal and either 5 μg of the vector alone, 3 μg of the vector alone, and 2 μg of a Myc-tagged constitutively active form of the PI3K p110 catalytic subunit (Myr-p110) or 2 μg of Myr-p110 and 3 μg N17Rac and assayed for their ability to invade Matrigel as described above. The data shown are the mean values (±SD) of a representative experiment done in triplicate. The expression of the transfected cDNAs was confirmed by Western blotting and is shown below each graph.

the invasion of the MDA-MB-435 cells suggests that other PI3K downstream effectors, in addition to Rac, are important for invasion in these cells.

Involvement of PI3K in the α6β4-Dependent Migration of Invasive Colon Carcinoma Cells

We wished to confirm that the activation of PI3K by α6β4 in the MDA-MB-435 transfectants also occurred in a carcinoma cell line that endogenously expresses the α6β4 integrin and that this activation was related to the invasive properties of the cell. For this purpose we chose clone A cells, an invasive colon carcinoma cell line that we have characterized extensively. Clone A cells express relatively high levels of the α6β4 integrin and no α6β1 integrin (Lee et al., 1992). Importantly, these cells use the α6β4 integrin as an adhesion receptor for laminin-1 (Lotz et al., 1990; Lee et al., 1992) in contrast to the MDA-MB-435/β4 transfectants. As shown in Figure 7A, the Matrigel invasion of clone A cells is inhibited by α6-specific antibodies.

Recently, we demonstrated that the α6β4 integrin performs an essential role in the migration of clone A cells on laminin-1 by promoting the formation and stabilization of filopodia and lamellae (Rabinovitz and Mercurio, 1997). Based on the data obtained with MDA-MB-435 cells, the prediction can be made that the α6β4-dependent formation of actin-containing motility structures is dependent on PI3K and that ligation of α6β4 stimulates PI3K activity in these cells. To examine this possibility, clone A cells were treated with wortmannin (100 nM) prior to their plating on laminin-1, and their behavior was then monitored by video microscopy. Wortmannin had no effect on the attachment of clone A cells to laminin or on their initial spreading. However, wortmannin inhibited the formation of lamellae by 80% (Figure 7B). Interestingly, the effect of wortmannin on the formation of lamellae was very similar to the effect of a function-blocking α6 antibody that recognizes only the α6β4 integrin in these cells (Figure 7C).

To examine whether α6β4 activates PI3K in clone A cells, we performed in vitro kinase assays on cell extracts prepared from cells that had attached to laminin-1 in the presence of either nonspecific IgG or an α6-specific antibody. As shown in Figure 8, PI3K activity was increased after attachment to laminin-1, but this increase was inhibited when the α6β4 receptor was blocked

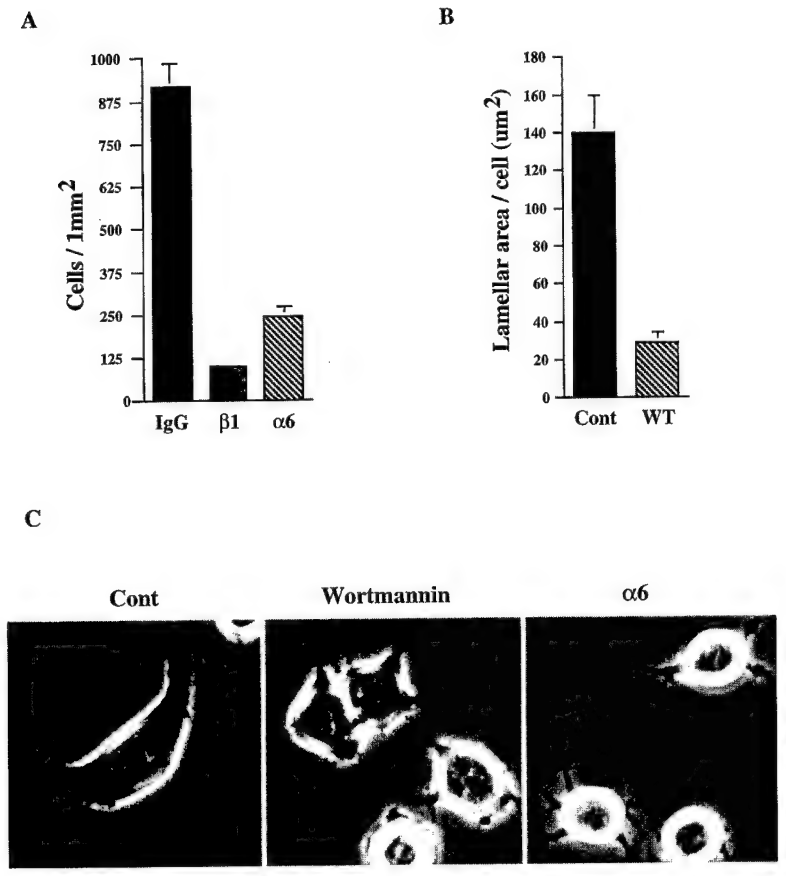

Figure 7. Analysis of α6β4 Function in an Invasive Colon Carcinoma Cell Line

(A) Clone A cells were assayed for their ability to invade Matrigel in the presence of monoclonal antibodies specific for the indicated integrin subunits. Cells were preincubated for 30 min in the presence of antibodies before addition to the Matrigel-coated wells. After 24 hr at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Experimental Procedures. The data shown are the mean values (±SD) of a representative experiment done in duplicate.

(B) Clone A cells were allowed to adhere to laminin-1 in the absence or presence of wortmannin (100 nM) for 45 min. Images were obtained at this time using a Nikon Diaphot 300 inverted microscope with phase contrast optics. This microscope was connected to a CCD camera (Dage-MTI), a frame-grabber (Scion), and a 7600 Power Macintosh computer to capture the images. The lamellar area for the cells in each condition was determined using IPlab Spectrum image analysis software. The data shown are the mean values (±SEM) for ≥50 cells. WT, wortmannin.

(C) Clone A cells were allowed to adhere to laminin-1 in the absence or presence of wortmannin (100nM) or the α6-specific antibody 2B7, as indicated, for 45 min. Representative images from cells in each condition are shown.

IgG, non-specific antibody; β1, MAb 13; α6, 2B7; Cont, control.

by the α6 antibody. Although clone A cells still adhere to laminin-1 in the presence of the α6 antibody using the α2β1 integrin (Lotz et al., 1990; Lee et al., 1992; Figure 7C), there was no increase in PI3K activity in these cells compared to cells maintained in suspension. Therefore, a differential ability of the α6β4 and β1 integrins to activate PI3K is evident in clone A cells, as was observed in the MDA-MB-435/β4 transfectants. In summary, α6β4 is required for PI3K activation and formation of lamellae in response to laminin-1 attachment in clone A cells, functions that are required for the invasion of these cells.

Discussion

Our results establish that the coupling of a specific integrin, α6β4, to the PI3K signaling pathway promotes the invasion of carcinoma cells. In addition, we demonstrate specificity in integrin activation of this lipid kinase pathway because α6β4 activated PI3K better than α6β1 and other β1 integrins in the cells examined. An essential role for PI3K in invasion constitutes a novel function for this kinase and implies that downstream effectors of PI3K are critical for the invasive process. We provide evidence, in fact, that the small GTP-binding protein Rac is downstream of PI3K in the cells examined and that it is involved in invasion. In contrast, the serine/threonine kinases Akt and S6K do not contribute to the invasive process even though they are regulated by PI3K and are activated by the α6β4 integrin. Collectively, our findings provide a mechanism for the involvement of α6β4 in promoting carcinoma invasion and reveal a specific PI3K signaling pathway that is essential for this process.

Invasion is a defining event in carcinoma progression. In general, this process represents the ability of epithelial cells to acquire a mesenchymal phenotype characteristic of the breast and colon carcinoma cells used in this study. Numerous studies have demonstrated that the progression to invasive carcinoma involves loss of function of adhesion molecules involved in maintenance of the epithelial phenotype, namely, cadherins and catenins. These studies support this epithelial–mesenchymal transition model (Takeichi, 1993; Birchmeier et al., 1995). An issue that has been less clear, however, is an understanding of the mechanisms used by mesenchymal-like carcinoma cells to invade through basement membranes and stroma. Specifically, the role of epithelial integrins in invasion has been difficult to study because the progression to invasive carcinoma does not always involve gross alterations in their expression. More likely, the transition from a polarized epithelium to invasive cancer involves changes in integrin function that contribute to the invasive process as exemplified by our work on the α6β4 integrin. Although this integrin contributes to the polarized phenotype of epithelial cells through its ability to form stable adhesive contacts at the basal cell surface (Dowling et al., 1996; Vanderneut

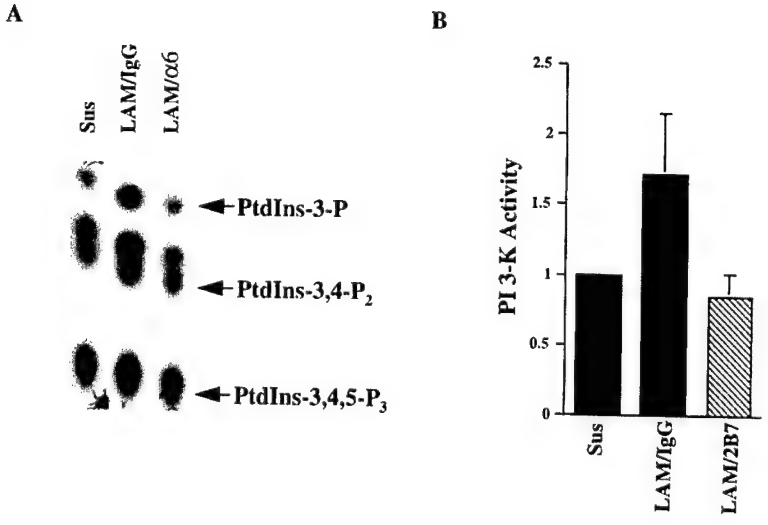

Figure 8. Analysis of PI3K Activity in Clone A Cells

(A) Clone A cells were maintained in suspension or allowed to adhere to laminin-1 in the presence of nonspecific IgG or α6-specific antibodies for 45 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine MAb 4G10 and Protein A sepharose for 3 hr. After washing, the beads were resuspended in kinase buffer and incubated for 10 min at room temperature. The labeled lipids were resolved by thin layer chromatography. The D3-phosphoinositides are indicated by arrows.

(B) The amount of radiolabeled PtdIns-3,4,5-P_3 was determined for each condition by densitometry. The adhesion-dependent levels of PtdIns-3,4,5-P_3 were compared to the level observed for the cells that were maintained in suspension, which was given the value of 1. The value from this ratio was determined to be the relative PI3K activity stimulated by adhesion. The data shown are the mean values (±SEM) from two experiments.

et al., 1996), the many pathological studies that have associated α6β4 with invasion suggested that its functions are also important for the invasive phenotype (Rabinovitz and Mercurio, 1996). We substantiated this possibility initially by showing that expression of α6β4 increased the invasion of both colon (Chao et al., 1996) and breast carcinoma cells (Figure 2A). Importantly, we now establish a mechanism for this increase by demonstrating that the α6β4 integrin activates a PI3K signaling pathway in carcinoma cells that is necessary for the invasive ability of these cells and that this α6β4-mediated pathway is linked to cell motility. Although protease activity is essential for invasion, no differences in protease expression or localization were observed between the mock and β4 transfectants of MDA-MB-435 cells (K. O'Connor, unpublished observation). Based on these findings, we postulate that the α6β4 integrin is critical for invasion because it promotes carcinoma motility through a PI3K-dependent pathway.

A central role for PI3K and its lipid products in carcinoma progression is indicated by our findings that wortmannin and dominant interfering p85 subunits of PI3K inhibited invasion of MDA-MB-435 cells and that a constitutively active p110 subunit of PI3K increased their invasion. The involvement of a PI3K-dependent signaling pathway in invasion adds to previous data that have implicated PI3K in tumor-promoting functions such as transformation, cell survival, anchorage-independent growth, and motility (Chang et al., 1997; Derman et al., 1997; Dudek et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997). Interestingly, integrins have been implicated in many of these processes as well (Meredith et al., 1993; Frisch and Francis, 1994; Huttenlocher et al., 1995). However, how such PI3K-dependent pathways are influenced by integrin signaling is an area that has not been well explored. Recent reports that MDCK cell adhesion to collagen (Khwaja et al., 1997) and COS cell adhesion to fibronectin (King et al., 1997) can increase the levels of PtdIns-3,4,5-P_3 and PtdIns-3,4-P_3 suggested that integrins regulate PI3K. We now provide evidence that integrins can differ in their ability to activate PI3K based on the preferential activation of PI3K by α6β4 compared to other β1 integrins in both the MDA-MB-435 and clone A cells and that this difference is linked to a specific cellular response.

Our data on the PI3K-dependent formation of lamellae in clone A cells indicate that the localized regulation of PI3K activity by α6β4 may provide an efficient mechanism for targeting downstream functional effects of this kinase. The migration of invasive carcinoma cells involves the dynamic formation of actin-containing motility structures such as lamellae and filopodia. In fact, we have shown recently that α6β4 is localized in lamellae and filipodia of clone A cells and that the migration of these cells on laminin-1 involves the α6β4 integrin–dependent formation of these structures (Rabinovitz and Mercurio, 1997). An important finding in the present study is that the formation of these lamellae is dependent on PI3K. Quite surprisingly, wortmannin inhibited the formation of lamellae but had little effect on the adhesion of these cells. These observations implicate a rather specific role for α6β4-regulated PI3K activity in inducing the formation of actin-containing motility structures in carcinoma cells. The lipid products of PI3K, the D3 phosphoinositides, could play a direct role in their formation because they can bind to a number of proteins that regulate actin assembly (Hartwig et al., 1996; Lu et al., 1996), and they have been shown to contribute to filopodial actin assembly in platelets (Hartwig et al., 1996).

An issue that arises from the data presented is the mechanism by which the α6β4 integrin activates PI3K. The preferential activation of PI3K by α6β4 compared to α6β1, as well as other β1 integrins, in the MDA-MB-435 cells suggests that the mechanism by which α6β4 activates PI3K differs either quantitatively or qualitatively from these integrins. We know that the β4 cytoplasmic domain is required for PI3K activation because a cytoplasmic domain mutant of the β4 subunit failed to increase PI3K activity upon ligation. Although the

sequence of the β4 cytoplasmic domain is different from other integrin β subunits, it does not contain the consensus sequence for p85 binding via SH2 domains, YMXM (Cantley and Songyang, 1994), thus diminishing the possibility of a direct association with PI3K. In fact, we have not been able to detect such an association in our experiments. More likely, signaling intermediates are involved. In keratinocytes, for example, the β4 cytoplasmic domain can bind to the adaptor protein Shc and link the α6β4 integrin to the activation of Ras and downstream MAPK pathways (Mainiero et al., 1997). Although MAPK does not appear to be essential for the invasion of MDA-MB-435 cells even though its activity is stimulated by α6β4 ligation, the issue of whether α6β4 activation of Ras itself is involved in PI3K activation is relevant because Ras has been shown to activate PI3K (Rodriguez-Viciana et al., 1994, 1996).

One downstream effector of PI3K that we demonstrate is involved in carcinoma invasion is the small GTP-binding protein Rac (Nobes and Hall, 1995). This observation supports the previous reports that both constitutively active Rac and Tiam-1, a GDP-dissociation stimulator for Rac, can induce the invasion of lymphoid cells (Habets et al., 1994; Michiels et al., 1995). Interestingly, neither constitutively active Rac nor constitutively active Rho and Cdc42 were able to increase invasion of MDA-MB-435 cells, suggesting that activation of other PI3K dependent pathways is also required in these cells. Given the potential importance of Rac to invasion, subsequent studies on the α6β4-PI3K pathway will focus on downstream effectors of Rac. One downstream effector of Rac that we have determined is not involved in invasion is p70 S6K (Chou and Blenis, 1995, 1996). Although p70 S6K is activated by α6β4 ligation in the MDA-MB-435 cells, rapamycin, which blocks p70 S6K activation (Chung et al., 1992; Kuo et al., 1992; Price et al., 1992), did not inhibit invasion. Other possible effectors of Rac are the family of related p21-activated serine/threonine kinases (PAK1, -2, -3) that bind to and are activated by both Rac and Cdc42 (Manser et al., 1994; Martin et al., 1995). The current data on the role of the PAKs in mediating the effects of Rac are conflicting (Harden et al., 1996; Joneson et al., 1996; Lamarche et al., 1996; Westwick et al., 1997). However, these kinases and their downstream effector JNK can mediate actin rearrangements in some cell types and, for this reason, could contribute to the motile events required for invasion. This possibility is interesting because results from primary keratinocytes suggest that the α6β4 integrin can activate JNK in a Rac-dependent manner and that this activity is inhibited by wortmannin (Mainiero et al., 1997).

The PI3K signaling pathways involved in invasion appear distinct from those involved in PI3K-dependent cell survival. Even though the Akt kinase is activated by α6β4 ligation, it is not required for invasion of the breast carcinoma cells we examined. In fact, expression of a constitutively active form of Akt actually inhibited the invasion of these cells, most likely because activated Akt uses the D3-phosphoinositide products of PI3K at the expense of those PI3K-dependent pathways that are involved in invasion. A fascinating problem is raised by these observations because carcinoma progression involves both tumor cell invasion and survival. The recent demonstration that Akt is required for the survival of several cell types (Dudek et al., 1997; Kennedy et al., 1997), coupled with our finding that PI3K is required for invasion, suggests that two essential functions of progression may require the products of PI3K and that the balance between the use of these pathways may impact tumor cell invasion or survival.

In summary, we have identified a specific integrin-mediated pathway involving PI3K that promotes carcinoma invasion. This pathway involves the small GTP-binding protein Rac. These findings are particularly important because they suggest that this PI3K signaling pathway is a potential target for inhibiting tumor spread.

Experimental Procedures

Cells

The MDA-MB-435 breast carcinoma cell line was obtained from the Lombardi Breast Cancer Depository at Georgetown University. The MDA-MB-435 cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (GIBCO) and 1% penicillin-streptomycin (GIBCO). Clone A cells were grown in RPMI supplemented with 25 mM HEPES (RPMI-H), 10% fetal calf serum, and 1% penicillin-streptomycin.

The cloning of the human β4 cDNA, the construction of the β4 cytoplasmic domain deletion mutant (β4-ΔCYT), and their insertions into the pRc/CMV (β4) and pcDNA3 (β4-ΔCYT) eukaryotic expression vectors, respectively, have been described previously (Clarke et al., 1995). The vectors containing the full-length and mutant β4 cDNAs, as well as the pcDNA3 vector alone, were transfected into the MDA-MB-435 cell line using Lipofectin (GIBCO) according to the manufacturer's instructions. Neomycin-resistant cells were isolated by selective growth in medium containing G418 (0.6 mg/ml; GIBCO). The stable transfectants were pooled and populations of cells that expressed the human β4 subunit on the cell surface were isolated by FACS. A human β4 integrin–specific MAb, UM-A9 (obtained from Tom Carey), was used for this sorting and for subsequent analysis of the transfectants. The sorting was repeated sequentially to enrich for homogeneous populations of cells expressing high levels of the transfected β4 and β4-ΔCYT subunits on the cell surface. Subclones were isolated from these populations by FACS. Surface labeling and immunoprecipitation with A9 were done to confirm that the α6β4 heterodimer was expressed on these subclones.

Analysis of Integrin Surface Expression

The relative surface expression of integrin subunits on the mock and β4 transfectants of the MDA-MB-435 cells was assessed by flow cytometry. For this purpose, aliquots of cells (5×10^5) were incubated for 45 min at room temperature with RPMI-H and 0.2% BSA (RH/BSA) and the following integrin-specific Abs: 2B7 (α6; prepared in our laboratory); MAb 13 (β1; provided by Stephen Akiyama); A9 (β4; provided by Thomas Carey); as well as mouse IgG (Sigma). The cells were washed two times with RH/BSA and then incubated with goat $F(ab')_2$ anti-mouse IgG coupled to fluorescein (Tago) for 45 min at room temperature. After washing two times with RH/BSA, the cells were resuspended in the same buffer and analyzed by flow cytometry.

Invasion Assays

Matrigel invasion assays were performed as described (Shaw et al., 1996) using 6.5 mm Transwell chambers (8 μm pore size; Costar). Matrigel, purifed from the EHS tumor, was diluted in cold distilled water, added to the Transwells (2–10 μg/well), and dried in a sterile hood. The Matrigel was then reconstituted with medium for an hour at 37°C before the addition of cells. Cells were resuspended in serum-free medium containing 0.1% BSA, and cells were added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers. After 4–6 hr, the cells that had not invaded were removed from the upper face of the filters using cotton swabs, and the cells that had invaded to the lower surface of the filters were fixed in methanol and then stained with a 0.2% solution of

crystal violet in 2% ethanol. Invasion was quantitated by visual counting. The mean of five individual fields in the center of the filter where invasion was the highest was obtained for each well. In some assays, the cells were preincubated for 30 min before addition to the Matrigel-coated wells with either wortmannin (Ui et al., 1995), PD98059 (Dudley et al., 1995; Pang et al., 1995), or rapamycin (Chung et al., 1992; Kuo et al., 1992; Price et al., 1992). For assays that used the transient transfectants described below, the cells were fixed for 30 min in 4% paraformaldehyde and then stained with PBS containing 1 mg/ml bluo-gal (Boehringer Mannheim), 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide.

Kinase Assays

Cells were removed from their dishes with trypsin and washed twice with RH containing 0.2% heat-inactivated BSA. After washing, the cells were resuspended in the same buffer at a concentration of 2×10^6 cells/ml and incubated for 30 min with integrin-specific antibodies or in buffer alone. The cells were washed once, resuspended in the same buffer, and added to plates that had been coated overnight with either anti-mouse IgG or laminin-1. After a 30 min incubation at 37°C, the cells were washed twice with cold PBS and solubilized at 4°C for 10 min in a 20 mM Tris buffer (pH 7.4), containing 0.14 M NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, 5 μg/ml aprotinin, pepstatin, and leupeptin. Nuclei were removed by centrifugation at 12,000 × g for 10 min.

To assay PI3K activity, aliquots of cell extracts that contained equivalent amounts of protein were incubated for 3 hr at 4°C with either the anti-phosphotyrosine MAb 4G10 (UBI) or a p85 subunit–specific PI3K antibody and Protein A sepharose (Pharmacia). The sepharose beads were washed twice with solubilization buffer and twice with a 10 mM HEPES buffer (pH 7) containing 0.1 mM EGTA (kinase buffer). After removal of the last wash, the beads were resuspended in kinase buffer containing 10 μg of sonicated crude brain lipids (Sigma), 100 μM ATP, 25 mM $MgCl_2$, and 10 μCi [γ-^{32}P]ATP and incubated for 10 min at room temperature. The reaction was stopped by the addition of 60 μl 2N HCl and 160 μl of a 1:1 mixture of chloroform and methanol. Lipids were resolved by thin layer chromatography plates coated with potassium oxalate.

To assay MAP kinase, total cell extracts, prepared as described above, were resolved by electrophoresis on SDS-polyacrylamide gels (10%), transferred to nitrocellulose, and blotted with a phospho-specific ERK polyclonal antibody that recognizes the phosphorylated isoforms of ERK-1 and ERK-2 (New England BioLabs, Inc.). Immune complexes were detected using a secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (Amersham, Inc.). The blots were then stripped and reprobed with an ERK-1 antibody (provided by John Blenis) that recognizes both ERK-1 and ERK-2.

To assay Akt kinase activity, total cell extracts containing equivalent amounts of protein were precleared with a 1:1 mixture of Protein A/Protein G and then incubated with a polyclonal antibody that recognizes the C-terminal end of Akt (provided by Thomas Franke) for 3 hr at 4°C. After a 1 hr incubation with the Protein A/Protein G mixture, the beads were washed 3 times with solubilization buffer, once with H_20, and once with a 20mM HEPES buffer (pH 7.4) containing 10 mM $MgCl_2$ and 10 mM $MnCl_2$ (kinase buffer). After removal of the last wash, the beads were resuspended in 30 μl of kinase buffer containing 5 μM ATP, 1 mM DTT, 10 μCi [γ-^{32}P]ATP, and 1.5 μg of Histone H2B (Boehringer Mannheim) and incubated for 20 min at room temperature. The reaction was stopped by the addition of 5× Laemmli sample buffer and resolved by electrophoresis on SDS-polyacrylamide gels (12%).

To assay p70 S6K activation, total cell extracts, prepared as described above, were resolved by electrophoresis on SDS-polyacrylamide gels (8%), transferred to nitrocellulose, and blotted with a polyclonal antibody that recognizes the C-terminal end of p70 S6K (provided by John Blenis). Immune complexes were detected using a secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (Amersham, Inc.).

Transient Transfections

The constitutively active PI3K catalytic p110 subunit was a generous gift of Julian Downward, ICRF, London. The small GTP-binding proteins V14Rho, V12Rac, V12Cdc42, N17Rac, and N17Cdc42, in the pEBG vector, were a kind gift of Margaret Chou, University of Pennsylvania. The constitutively active Akt was kindly provided by Philip Tsichlis, Fox Chase Cancer Center, PA. The dominant negative PI3K p85 subunit was kindly provided by Brian Schaffhausen, Tufts University. The pCS2-(n)β-Gal was a gift from Sergei Sokol, Beth Israel Deaconess Medical Center.

Cells were cotransfected with 1 μg pCS2-(n)β-Gal and the cDNAs specified in the figure legends using Lipofectamine (GIBCO) according to manufacturer's instructions. Cells were harvested 24 hr after transfection and added to Matrigel invasion assays as described. Transfected cells were also plated in 48 wells to stain for β-galactosidase expression to determine transfection efficiency. The remaining cells were collected and extracted in RIPA buffer (phosphate buffered saline [pH 7.4] containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, and 5 μg/ml aprotinin, leupeptin, and pepstatin). These cell extracts were immunoprecipitated with HA- (Myr-Akt; Boehringer Mannheim), Myc- (Myr-p110 and p85; Oncogene Science), or GST- (V14Rho, V12Rac, V12Cdc42, N17Rac, N17Cdc42, and Dp85; Santa Cruz) specific antibodies, resolved by electrophoresis on SDS-polyacrylamide gels (10%), and transferred to nitrocellulose. The expression of the tagged proteins was detected by blotting with the same antibodies.

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Identification of Insulin Receptor Substrate 1 (IRS-1) and IRS-2 as Signaling Intermediates in the α6β4 Integrin-Dependent Activation of Phosphoinositide 3-OH Kinase and Promotion of Invasion

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Expression of the α6β4 integrin increases the invasive potential of carcinoma cells by a mechanism that involves activation of phosphoinositide 3-OH kinase (PI3K). In the present study, we investigated the signaling pathway by which the α6β4 integrin activates PI3K. Neither the α6 nor the β4 cytoplasmic domain contains the consensus binding motif for PI3K, pYMXM, indicating that additional proteins are likely to be involved in the activation of this lipid kinase by the α6β4 integrin. We identified insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the activation of PI3K by the α6β4 integrin. IRS-1 and IRS-2 are cytoplasmic adapter proteins that do not contain intrinsic kinase activity but rather function by recruiting proteins to surface receptors, where they organize signaling complexes. Ligation of the α6β4 receptor promotes tyrosine phosphorylation of IRS-1 and IRS-2 and increases their association with PI3K, as determined by coimmunoprecipitation. Moreover, we identified a tyrosine residue in the cytoplasmic domain of the β4 subunit, Y1494, that is required for α6β4-dependent phosphorylation of IRS-2 and activation of PI3K in response to receptor ligation. Most importantly, Y1494 is essential for the ability of the α6β4 integrin to promote carcinoma invasion. Taken together, these results imply a key role for the IRS proteins in the α6β4-dependent promotion of carcinoma invasion.

Cell adhesion molecules play an important role in normal epithelia, and changes in their expression and function contribute to the progression of epithelial cells to invasive, metastatic carcinoma. For example, cell-cell interactions in many tumors are altered through a quantitative decrease in cadherin expression, which reduces intercellular adhesion (5). This disruption of cell-cell adhesion is permissive for increased cell motility. Cell adhesion can also be modified through qualitative changes in receptor function that promote the dynamic adhesion that is required for motile, invasive cells (31). In recent years, a significant amount of evidence to suggest that the α6β4 integrin is a member of this category of adhesion receptors has accumulated. Specifically, the expression of this integrin receptor is maintained in carcinoma cells but it functions in a manner distinct from its role in normal epithelial cells (55). The involvement of the β4 integrin subunit in carcinoma cell biology was initially suggested by its identification as a tumor-related antigen expressed in metastatic cancer (22). Since then, many studies have reported a strong association of β4 expression with solid-tumor progression. For example, the β4 subunit is not expressed in the normal thyroid but its expression correlates with the progression to invasive thyroid carcinoma (62). The β4 subunit is also expressed in androgen receptor-negative invasive prostate carcinomas and at the leading edges of invading gastric carcinomas (6, 75). Moreover, expression of the β4 subunit correlates with a poor prognosis in patients with squamous cell, breast, and colon carcinomas (23, 71, 82). These correlative data have been supported more recently by functional studies that have provided mechanistic insight into how the α6β4 integrin contributes to tumor progression. In previous studies, we demonstrated that the α6β4 integrin can increase the invasive potential of breast carcinoma cells, a finding that has been confirmed for other cell types as well (12, 21, 64, 67). Furthermore, expression of the α6β4 receptor increases the survival of p53 mutant carcinoma cells (2, 74). Given that invasion and survival are two critical functions of metastatic cells, it is important to understand in more detail the mechanism of action of the α6β4 integrin in tumor cells.

In normal epithelia, the α6β4 integrin functions as a receptor for the laminin family of extracellular matrix proteins and mediates the stable attachment of epithelial cells to the underlying basement membrane (7, 41). Many studies, including those involving knockout of the β4 subunit, have substantiated the importance of the adhesive contributions of the α6β4 integrin to normal epithelial function (19, 81). In the absence of the β4 subunit, and more specifically the β4 cytoplasmic domain, a lethal blistering of the epithelium, which is known as epidermolysis bullosa, occurs (19, 81). In carcinoma cells, the α6β4 integrin also functions as a laminin receptor and the β4 subunit interacts with the actin cytoskeleton to promote the formation of actin-rich structures that are important for cell motility (54, 56). However, in addition to its mechanical involvement in mediating adhesive interactions, the α6β4 integrin activates intracellular signaling pathways that are essential

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for the ability of this receptor to promote tumor progression (51, 52, 64). For example, our analysis of the mechanism involved in the α6β4-dependent promotion of invasion revealed that phosphoinositide 3-OH kinase (PI3K) activation by the receptor is essential for this function (64). The ability of the α6β4 integrin to promote PI3K activation is greater than that observed for other β1 integrins, which supports the increased potential of this integrin to promote carcinoma invasion. Activation of PI3K by the α6β4 integrin is also required for the ability of this integrin to promote carcinoma cell survival through the activation of the Akt kinase (2, 3, 74).

PI3K is a lipid kinase that phosphorylates the D3 position of inositol lipids to form the products phosphatidylinositol (PtdIns)-3-P, PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3 (76). These D3 phosphoinositides are expressed at very low levels in unstimulated cells, but their levels are increased in response to many different stimuli, supporting their role as second messengers. A major function of the D3 phosphoinositides is to bind and recruit signaling molecules to the plasma membrane, where they can interact with other regulatory and effector molecules (76). The involvement of PI3K in carcinoma cell biology has been proposed from both direct and indirect evidence. As mentioned above, PI3K activity promotes carcinoma invasion and survival, and it has also been implicated in promoting anchorage-independent growth (20, 34, 35, 64). An avian sarcoma virus that encodes the catalytic subunit of PI3K transforms chicken embryo fibroblasts, suggesting that PI3K can also play a role in the early stages of tumor initiation (11). Finally, PTEN, a lipid phosphatase that regulates the levels of the PI3K lipid products, is frequently mutated or deleted in tumors (9, 18). The identification of the PTEN gene as a tumor suppressor gene demonstrates the importance of tightly regulating the activity of PI3K. In light of these findings, the relevance of determining how the α6β4 integrin activates the PI3K signaling pathway is evident.

The α6β4 integrin is distinct from other integrin receptors because the β4 subunit contains a 1,000-amino-acid cytoplasmic domain (27, 70, 72). This large intracellular domain is important for many of the known α6β4-dependent functions. For example, the β4 cytoplasmic domain is essential for hemidesmosome formation in normal epithelial cells and it is required for promoting carcinoma cell invasion (44, 64). In the absence of the β4 cytoplasmic domain, the α6β4 receptor is not capable of activating PI3K or other signaling pathways that have been shown to be activated by this integrin, including the mitogen-activated protein kinase (MAPK) pathway (64). Although a number of proteins that interact with the β4 cytoplasmic domain in hemidesmosomes have been identified, very little is known about the structural requirements for signaling by this integrin or the specific interactions that occur with downstream effectors to initiate signals (48, 57). Recently, the binding site in the β4 cytoplasmic domain for the adapter protein Shc, which recruits Grb2 and Sos to promote Ras activation, has been identified (16, 43). However, the binding motif for the p85 regulatory subunit of PI3K, YMXM, is not present in the β4 cytoplasmic domain, which suggests that alternative mechanisms are required to recruit this lipid kinase (10).

In the present study, we have examined the mechanism by which the α6β4 integrin activates the PI3K signaling pathway. In light of the fact that the β4 subunit cytoplasmic domain does not contain a binding site to directly interact with PI3K, we sought to identify the intermediate proteins that are responsible for recruiting PI3K to the plasma membrane in response to α6β4 ligation. We identified two members of the insulin receptor substrate (IRS) family, IRS-1 and IRS-2, which are specific mediators of α6β4-dependent activation of PI3K (84). In addition, we investigated the structural requirements of the β4 subunit cytoplasmic domain for PI3K activation. Through this analysis we identified a specific tyrosine residue in the β4 subunit, Y1494, that is required for α6β4-dependent activation of PI3K and, importantly, for the ability of this integrin receptor to promote carcinoma invasion.

MATERIALS AND METHODS

Cells and antibodies. MDA-MB-435 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker) supplemented with 10% fetal calf serum (Sigma), 1% penicillin-streptomycin (Gibco), and 1% GlutaMax (Gibco). T47D cells were grown in DMEM supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 1% GlutaMax, and 5 μg of insulin (Gibco)/ml. The rat monoclonal antibody that recognizes the α6-integrin subunit (135-13C) was a gift from Rita Falcioni, and the mouse monoclonal antibody that recognizes the β4-integrin subunit (UM-A9) was purchased from Ancell. The IRS-1-specific polyclonal antibody was purchased from Santa Cruz Biotechnology. The IRS-2-specific polyclonal antibody and the 4G10 phosphotyrosine-specific monoclonal antibody were purchased from Upstate Biotechnology Inc. The RC-20 biotinylated-phosphotyrosine-specific monoclonal antibody was purchased from Transduction Labs. The p85-specific polyclonal antiserum was a gift from Alex Toker.

Integrin clustering. Cells were removed from their dishes with trypsin and washed twice with RPMI medium containing 25 mM HEPES (RH) and 0.1% heat inactivated bovine serum albumin (BSA; RH-BSA). After being washed, the cells were resuspended in the same buffer at a concentration of 2×10^6 cells/ml and incubated for 30 min with integrin-specific antibodies or in buffer alone. The cells were washed once, resuspended in the same buffer, and added to plates that had been coated overnight with anti-mouse immunoglobulin G (IgG). After a 30-min incubation at 37°C, the cells were washed twice with cold phosphate-buffered saline (PBS) and solubilized at 4°C for 10 min in a 20 mM Tris buffer, pH 7.4, containing 0.14 M NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 5 μg of aprotinin, pepstatin, and leupeptin/ml. Nuclei were removed by centrifugation at 12,000 × *g* for 10 min. For laminin attachment assays, cells were added to plates (100 mm in diameter) that had been coated overnight with 150 μg of laminin-1 and incubated for 45 min at 37°C.

Immunoprecipitation and immunoblotting. Aliquots of cell extracts containing equivalent amounts of protein were incubated for 3 h at 4°C with antibodies and protein A- or protein G-Sepharose (Pharmacia) with constant agitation. The beads were washed three times in the extraction buffer. Laemmli sample buffer was added to the samples, which were then incubated at 100°C for 4 min. Immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filters. The filters were blocked for 1 h using a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl and 0.05% Tween 20 (TBST) and 5% (wt/vol) Carnation dry milk. The filters were incubated for 1 h in the same buffer containing primary antibodies. After three 10-min washes in TBST, the filters were incubated for 1 h in blocking buffer containing horseradish peroxidase (HRP)-conjugated secondary antibodies. After three 10-min washes in TBST, proteins were detected by enhanced chemiluminescence (Pierce). For RC-20 phosphotyrosine immunoblots, the filters were blocked for 1 h using a 10 mM Tris buffer, pH 7.5, containing 0.5 M NaCl and 0.1% Tween 20 (RC-20 buffer) and 2% (wt/vol) Carnation dry milk. The filters were washed briefly in RC-20 buffer and then incubated overnight at 4°C in RC-20 buffer containing 3% (wt/vol) BSA and a 1:500 dilution of the RC-20 antibody. After being washed, the filters were incubated for 1 h in blocking buffer containing HRP-conjugated streptavidin, and the proteins were detected by enhanced chemiluminescence.

PI3K kinase assay. To assay PI3K activity, aliquots of cell extracts that contained equivalent amounts of protein were incubated for 3 h at 4°C with antibodies and protein A-Sepharose (Pharmacia). The Sepharose beads were washed twice with solubilization buffer and twice with a 10 mM HEPES buffer, pH 7, containing 0.1 mM EGTA (kinase buffer). After removal of the last wash,

the beads were resuspended in kinase buffer containing 10 μg of sonicated crude brain lipids (Sigma), 100 μM ATP, 25 mM $MgCl_2$, and 10 μCi of [γ-^{32}P]ATP and incubated for 10 min at room temperature. The reaction was stopped by the addition of 60 μl of 2 N HCl and 160 μl of a 1:1 mixture of chloroform and methanol. Lipids were resolved by using thin-layer chromatography plates coated with potassium oxalate.

Site-directed mutagenesis. The cloning of the human wild-type β4 cDNA (β4D) and its transfection into the MDA-MB-435 cell line have been described previously (13, 64). Tyrosine residues 1257 and 1494 in the β4 subunit were mutated to phenylalanine residues using the Quickchange site-directed mutagenesis kit (Stratagene). Briefly, overlapping primers containing the desired mutations were used to amplify the β4 cDNA and vector by PCR and the resulting point mutations were confirmed by dideoxy sequencing. The vectors containing the mutant β4 cDNAs were transfected into the MDA-MB-435 cell line using Lipofectamine (Gibco) according to the manufacturer's instructions. Neomycin-resistant cells were isolated by selective growth in medium containing G418 (0.8 mg/ml; Gibco). The stable transfectants were pooled, and subclones of cells that expressed the mutant β4 subunits on the cell surface were isolated by fluorescence-activated cell sorting (FACS). The human β4 integrin-specific monoclonal antibody, UM-A9 (Ancell), was used for this sorting and for subsequent analysis of the transfectants.

Analysis of integrin surface expression. The relative surface expression of the β4-integrin subunit on the transfected MDA-MB-435 subclones was assessed by flow cytometry. For this purpose, aliquots of cells (5×10^5) were incubated for 45 min at room temperature with RH-BSA and either the β4-specific antibody or nonspecific mouse IgG (Sigma). The cells were washed two times with RH-BSA and then incubated with goat anti-mouse IgG coupled to Cy2 (Jackson Immunoresearch) for 45 min at room temperature. After being washed two times with RH-BSA, the cells were resuspended in PBS and analyzed by flow cytometry.

Invasion assay. Matrigel invasion assays were performed as described previously (63, 64) using 6.5-mm-diameter Transwell chambers (8-μm pore size; Costar). Matrigel purified from the Englebreth-Holm-Swarm tumor was diluted in cold distilled water, added to the Transwells (5 μg/well), and dried in a sterile hood. The Matrigel was then reconstituted with medium for 1 h at 37°C before the addition of cells. Cells (0.5×10^5) were resuspended in serum-free DMEM containing 0.1% BSA, and cells were added to each well. Conditioned NIH 3T3 medium was added to the bottom wells of the chambers. After 4 h, the cells that had not invaded were removed from the upper faces of the filters using cotton swabs and the cells that had invaded to the lower surfaces of the filters were fixed in methanol and then stained with a 0.2% solution of crystal violet in 2% ethanol. Invasion was quantitated by visual counting. The mean of five individual fields in the center of the filter, where invasion was the highest, was obtained for each well.

Adhesion assays. Adhesion assays were performed as described previously (63). Briefly, multiwell tissue culture plates (11.3 mm in diameter) were coated overnight at 4°C with 0.2 ml of PBS containing either murine laminin-1 (20 μg/ml) or rat collagen I (20 μg/ml). The wells were then washed with PBS and blocked with RH-BSA. Cells (10^5) were resuspended in RH-BSA and added to the protein-coated wells. After a 60-min incubation at 37°C, the wells were washed three times with RH at 37°C, fixed for 15 min with methanol, and stained with a 0.2% solution of crystal violet in 2% ethanol. The crystal violet stain was solubilized with a 1% solution of SDS, and adhesion was quantitated by measuring the absorbance at 595 nm.

RESULTS

Identification of IRS-1 and IRS-2 as intermediates in the activation of PI3K by the α6β4 integrin. In previous work, we demonstrated that ligation of the α6β4 integrin promotes significantly more PI3K activity than ligation of the α6β1 integrin or other β1 integrins (64). PI3K is activated by recruitment of the p85 regulatory subunit to phosphotyrosine-containing binding motifs (pYMXM) (10). Neither the α6- nor the β4-subunit cytoplasmic domain contains the p85 consensus binding motif, which suggests that additional intermediate proteins are most likely involved in the α6β4-dependent activation of PI3K. To identify these intermediates, we analyzed the profile of phosphoproteins that associate with PI3K after α6β4 ligation. To do so, cell extracts from mock- (MDA-MB-435/mock) and β4-transfected (MDA-MB-435/β4) MDA-MB-435 cells

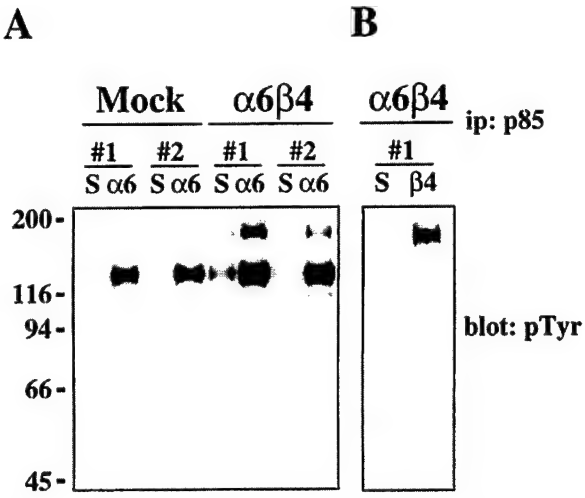

FIG. 1. Analysis of PI3K-associated proteins. Two subclones each (#1 and #2) of the MDA-MB-435/mock and the MDA-MB-435/β4 transfectants were maintained in suspension or incubated with either α6- (A) or β4-specific (B) antibodies and allowed to adhere to either anti-rat IgG- or anti-mouse IgG-coated plates, respectively, for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with a polyclonal antiserum specific for the p85 subunit of PI3K and protein A-Sepharose for 3 h. The immune complexes were resolved by SDS–8% PAGE and then immunoblotted with phosphotyrosine-specific antibody RC-20. Mock, MDA-MB-435 cells transfected with the empty vector; α6β4, MDA-MB-435 cells transfected with the full-length β4 subunit; S, cells maintained in suspension; α6, cells clustered with an α6-specific antibody (135-13C); β4, cells clustered with a β4-specific antibody (UM-A9). ip, immunoprecipitation.

that had been clustered with α6-specific antibody 135-13C were immunoprecipitated with a p85-specific antiserum and the associated proteins were detected by immunoblotting with phosphotyrosine-specific antibody RC-20. As shown in Fig. 1A, ligation of both α6β1 and α6β4 resulted in the interaction of PI3K with a 130-kDa phosphoprotein. However, an additional 180-kDa phosphoprotein coimmunoprecipitated with PI3K in both of the MDA-MB-435/β4 subclones after ligation with α6-specific antibodies. To confirm that this 180-kDa protein was specific for α6β4-dependent activation of PI3K, the MDA-MB-435/β4 transfectants were clustered with β4-specific antibodies, which will not ligate α6β1, and the p85-associated proteins were analyzed. When α6β4 was clustered in the absence of α6β1 ligation, only the 180-kDa protein was observed to coimmunoprecipitate with PI3K (Fig. 1B). The association of PI3K with the 180-kDa phosphoprotein only after ligation of the α6β4 integrin suggests that there is a unique mechanism for PI3K activation by the α6β4 receptor that is not utilized by the α6β1 receptor.

To understand further the mechanism of α6β4-dependent PI3K activation, we sought to identify the 180-kDa protein that was phosphorylated on tyrosine and associated with PI3K in response to α6β4 ligation. Given the molecular mass of this phosphoprotein, we first investigated if this protein was a growth factor receptor. This possibility was supported by the fact that the α6β4 integrin can associate with ErbB2 (21). However, the p85-associated 180-kDa protein was not expressed on the cell surface, as indicated by a test for surface biotinylation (data not shown).

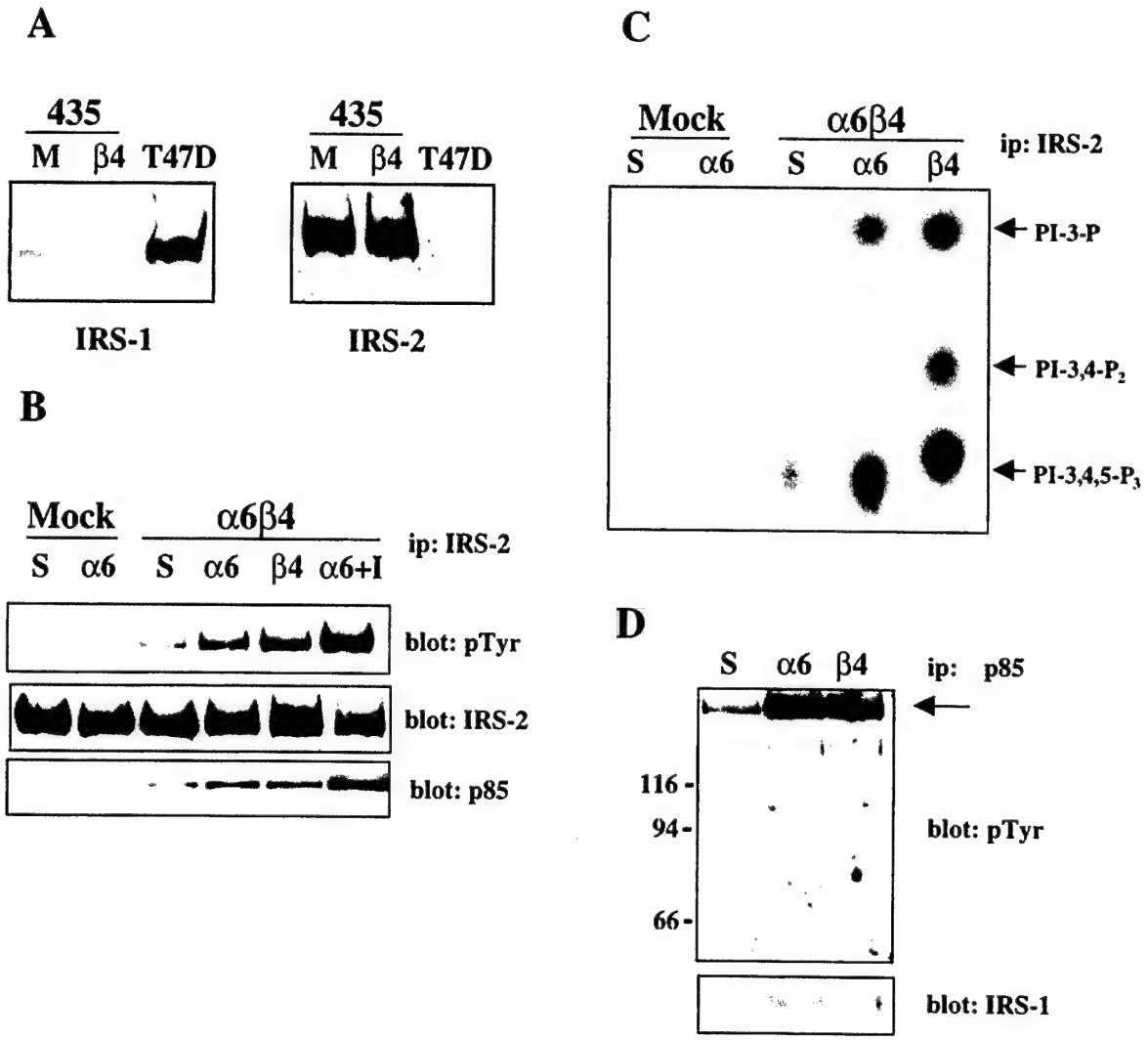

FIG. 2. Identification of IRS-1 and IRS-2 as α6β4-dependent PI3K-associated proteins. (A) Aliquots of cell extracts from the MDA-MB-435 transfectants and T47D breast carcinoma cells containing equivalent amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies specific for IRS-1 and IRS-2. 435, MDA-MB-435 cells; M, MDA-MB-435 cells transfected with the empty vector; β4, MDA-MB-435 cells transfected with the full-length β4 subunit. (B) MDA-MB-435 transfectants were maintained in suspension or incubated with either α6- or β4-specific antibodies and allowed to adhere to either anti-rat IgG- or anti-mouse IgG-coated plates, respectively, in the absence or presence of IGF-1 (100 ng/ml) for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with an IRS-2-specific polyclonal antibody and protein A-Sepharose for 3 h. The immune complexes were resolved by SDS–8% PAGE and then immunoblotted with RC-20 (top). The immunoblot was subsequently stripped and reprobed with IRS-2- (middle) and p85-specific (bottom) polyclonal antisera. ip, immunoprecipitation. (C) MDA-MB-435 transfectants were treated as described above, and aliquots of cell extracts that contained equivalent amounts of protein were incubated with an IRS-2-specific antibody and protein A-Sepharose for 3 h. After being washed, the beads were resuspended in kinase buffer and incubated for 10 min at room temperature. The phosphorylated lipids were resolved by thin-layer chromatography. Arrows, D3 phosphoinositides. (D) T47D cells were treated as described above, and aliquots of cell extracts that contained equivalent amounts of protein were incubated with a p85-specific antibody and protein A-Sepharose for 3 h. The immune complexes were resolved by SDS–8% PAGE and then immunoblotted with RC-20 (top). The immunoblot was subsequently stripped and reprobed with an IRS-1-specific polyclonal antibody (bottom). Mock, MDA-MB-435 cells transfected with the empty vector; α6β4, MDA-MB-435 cells transfected with the full-length β4 subunit; S, cells maintained in suspension; α6, cells clustered with an α6-specific antibody (135-13C); β4, cells clustered with a β4-specific antibody (UM-A9).

Next, we investigated the IRS adapter proteins. The IRS family members, which include IRS-1, IRS-2, IRS-3, and IRS-4, are 170- to 180-kDa proteins (except IRS-3, which is 60 kDa) that function as intermediate docking proteins downstream of the insulin and insulin-like growth factor 1 (IGF-1) receptors, as well as a number of cytokine receptors (84). In addition, the α5β1 integrin can promote IRS-1 phosphorylation in adipocytes (24). Importantly, the IRS proteins contain several PI3K binding sites, and these adapters are known to be involved in the activation of PI3K downstream of several of the receptors mentioned above (30, 33, 45, 69, 78, 86). To determine if the 180-kDa protein that coimmunoprecipitated with PI3K was an IRS family member, we first analyzed the expression of each IRS homolog in MDA-MB-435 cells. As shown in

Fig. 2A, MDA-MB-435 cells express very low levels of IRS-1 but express high levels of IRS-2. IRS-3 and IRS-4 were not detected in these cells (data not shown). To investigate the potential involvement of IRS-2 in the α6β4-dependent activation of PI3K, cell extracts from MDA-MB-435/mock and MDA-MB-435/β4 cells that had been clustered with α6-specific antibodies were assayed for IRS-2 phosphorylation. As shown in Fig. 2B, IRS-2 was phosphorylated on tyrosine in response to clustering with α6-specific antibodies in the MDA-MB-435/β4 transfectants but not in the MDA-MB-435/mock transfectants. In addition, ligation with a β4-specific antibody also increased the tyrosine phosphorylation of IRS-2. Most importantly, the p85 subunit of PI3K associated with IRS-2 after ligation of the α6β4 receptor (Fig. 2B, bottom). As a positive control for IRS-2 phosphorylation, the MDA-MB-435/β4 cells were treated with IGF-1, which increased the phosphorylation of IRS-2 and its association with PI3K (Fig. 2B). The MDA-MB-435/mock and -β4 transfectants express equivalent levels of IRS-2, and therefore the lack of IRS-2 phosphorylation after ligation of α6β1 in the MDA-MB-435/mock transfectants is not due to a relative difference in protein expression levels (Fig. 2A).

To confirm that PI3K is activated through IRS-2 in response to α6β4 ligation, in vitro kinase assays were performed on IRS-2 immune complexes. MDA-MB-435/mock and MDA-MB-435/β4 transfectants were clustered with α6- and β4-specific antibodies, and the cell extracts were immunoprecipitated with IRS-2 antibodies. The IRS-2 immunoprecipitates were assayed for their ability to phosphorylate crude brain phosphoinositides. As shown in Fig. 2C, ligation of the α6β4 integrin with both α6- and β4-specific antibodies resulted in a marked increase in PI3K activity associated with IRS-2, as demonstrated by the appearance of the PtdIns-3,4,5-P_3 lipid product. In contrast, ligation of α6β1 in the MDA-MB-435/mock transfectants resulted in minimal IRS-2-associated PI3K activity.

IRS-1 and IRS-2 have considerable structural homology and both homologs contain multiple binding sites for PI3K (84). To determine if IRS-1 can also be involved in the activation of PI3K by the α6β4 integrin, we used T47D breast carcinoma cells, which express high levels of IRS-1 and low levels of IRS-2 (Fig. 2A). As shown in Fig. 2D (top), ligation of the α6β4 receptor using either α6- or β4-specific antibodies increased the association of PI3K with a 170-kDa phosphoprotein. An IRS-1-specific antibody recognized this phosphoprotein (bottom). Based on these results we conclude that both IRS-1 and IRS-2 can function as intermediate signaling proteins in the activation of PI3K by the α6β4 integrin. The 130-kDa phosphoprotein that associates with PI3K in response to α6β1 ligation has not been conclusively identified.

Adhesion to laminin-1 promotes α6β4-dependent IRS phosphorylation. To confirm that the α6β4-dependent IRS signaling pathway that we identified by antibody clustering occurs in response to ligation of α6β4 by a natural extracellular matrix ligand, we assessed the phosphorylation of IRS-2 after adhesion of the MDA-MB-435/mock and MDA-MB-435/β4 transfectants to a laminin-1 substratum. As shown in Fig. 3, adhesion of the MDA-MD-435/mock transfectants to laminin-1 did not result in an increase in the tyrosine phosphorylation of IRS-2. Given that α6β1 is the major laminin receptor on the surfaces of these cells, these results confirm the data obtained using antibodies to cluster the α6β1 integrin (Fig. 2B). In contrast, adhesion of the MDA-MB-435/β4 transfectants to laminin-1 promoted the tyrosine phosphorylation of IRS-2 and the recruitment of PI3K to this adapter protein. Therefore, ligation of the α6β4 integrin by either laminin-1 or receptor-specific antibodies can activate the IRS-PI3K signaling pathway.

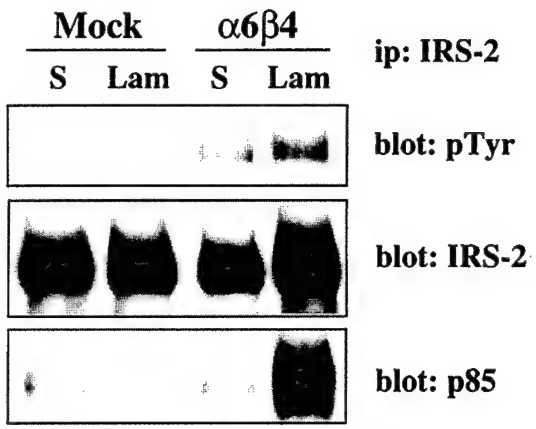

FIG. 3. α6β4-dependent IRS-2 phosphorylation in response to laminin-1 adhesion. MDA-MB-435 transfectants were maintained in suspension or allowed to adhere to laminin-1-coated plates for 45 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with an IRS-2-specific antibody and protein A-Sepharose for 3 h. The immune complexes were resolved by SDS–8% PAGE and then immunoblotted with RC-20 (top). The immunoblot was subsequently stripped and reprobed with IRS-2- (middle) and p85-specific (bottom) polyclonal antisera. Mock, MDA-MB-435 cells transfected with the empty vector; α6β4, MDA-MB-435 cells transfected with the full-length β4 subunit; S, cells maintained in suspension; Lam, cells adherent to a laminin substratum. ip, immunoprecipitation.

Analysis of tyrosine phosphorylation of the β4 subunit. Having identified IRS-1 and IRS-2 as signaling intermediates in the pathway utilized by the α6β4 integrin to activate PI3K, we next investigated the mechanism by which this integrin receptor activates this pathway. It had been previously demonstrated that the β4 subunit is phosphorylated on tyrosine after ligation of the α6β4 receptor (43). We confirmed this finding in our transfected MDA-MB-435/β4 cells. As shown in Fig. 4A, a time-dependent increase in the tyrosine phosphorylation of the β4 subunit was observed after ligation of the α6β4 receptor with α6-specific antibodies. Addition of sodium orthovanadate markedly increased the level of tyrosine phosphorylation, indicating that tyrosine phosphorylation of the β4 subunit is regulated by tyrosine phosphatases in these cells (Fig. 4A) (43). To evaluate the role of β4-tyrosine phosphorylation in the activation of PI3K by the α6β4 integrin, we analyzed the β4 cytoplasmic domain for tyrosine residues that were located within known consensus binding or phosphorylation motifs. We identified two tyrosines that were of potential interest, Y1257 and Y1494 (Fig. 3B). Both of these tyrosines are located within immune T-cell inhibitory motifs (ITIM) that have the consensus sequence of L/VXXpYXL/V and that were initially identified in immune cell inhibitory coreceptors (79). ITIM sites are involved in regulating B- and T-cell receptor signaling, and they have been characterized as binding motifs

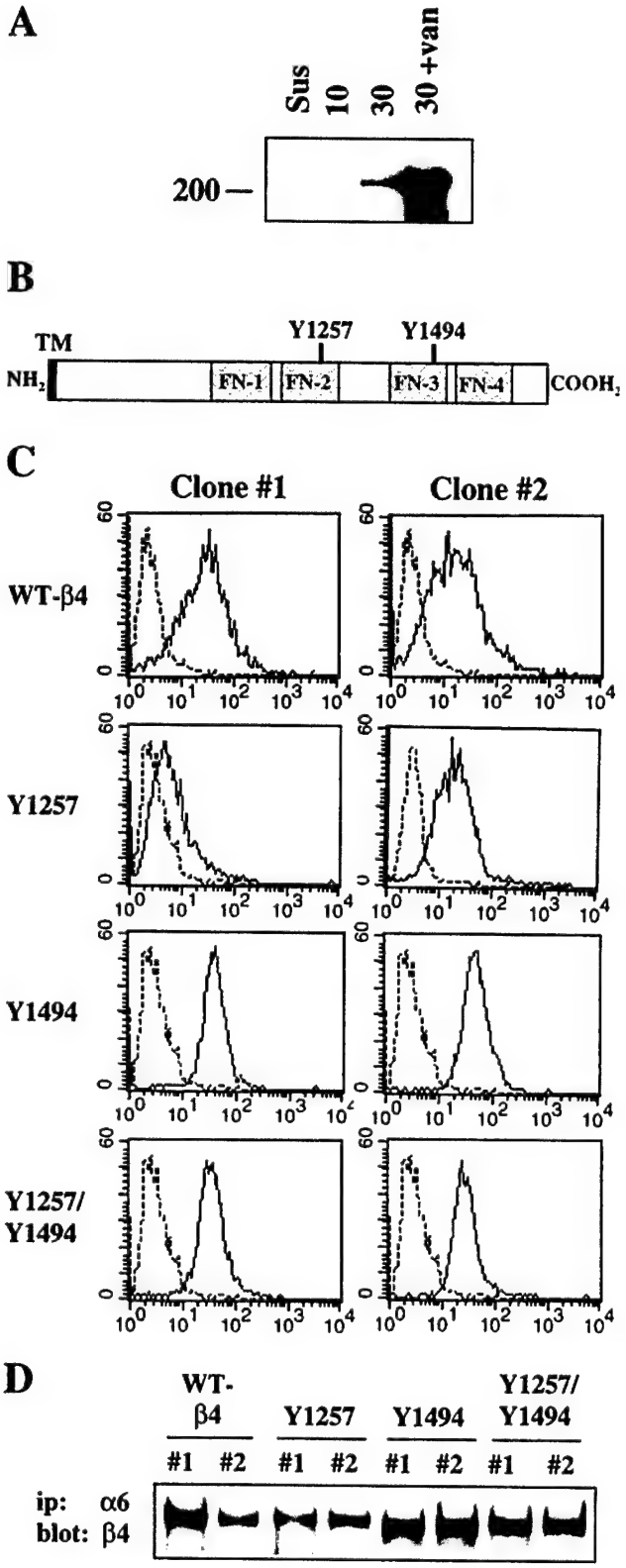

FIG. 4. Characterization of tyrosine mutants of the β4-integrin subunit. (A) MDA-MB-435/β4 transfectants were maintained in suspension or incubated with α6-specific antibodies and allowed to adhere to anti-rat IgG-coated plates in the absence or presence of 100 μM sodium orthovanadate (van) for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with a β4-specific antibody (UM-A9) and protein G-Sepharose for 3 h. The immune complexes were resolved by SDS–8% PAGE and then immunoblotted with RC-20. (B) Schematic of the β4-integrin subunit cytoplasmic domain, which indicates the tyrosine residues which were mutated to phenylalanine. TM, transmembrane domain. (C) Subclones of transfected MDA-MB-435 cells expressing the β4 subunit on the cell surface were isolated by FACS using a β4-specific antibody (UM-A9). MDA-MB-435 cells transfected with the wild-type (WT) and mutant human β4-integrin subunits were analyzed by flow cytometry using nonspecific mouse IgG (left peak) or UM-A9 (right peak). Shown are two representative subclones (clones 1 and 2) from each transfectant line. (D) Aliquots of cell extracts from the MDA-MB-435 transfectants were immunoprecipitated (ip) with an α6-specific antibody (135-13C). The immune complexes were resolved by SDS–6% PAGE and immunoblotted with a polyclonal antiserum that recognizes the C terminus of the β4 subunit.

for the SH2 domains of the SH2-containing tyrosine phosphatase 1 (SHP-1) and -2 protein tyrosine phosphatases and also for the SH2-containing inositol polyphosphate 5-phosphatase 1 (SHIP-1) and -2 lipid phosphatases (79).

To investigate the potential involvement of Y1257 and Y1494 in the α6β4-dependent activation of PI3K, Y1257 and Y1494 in the β4 subunit were individually mutated to phenylalanine residues. In addition, we also mutated both Y1257 and Y1494 to generate a double-ITIM-mutant β4 subunit (Y1257F/Y1494F). The mutant β4 subunits were stably expressed in the MDA-MB-435 cells, which lack endogenous β4 expression, and subclones expressing the β4 mutant proteins on the cell surface were isolated by FACS. As shown in the flow cytometry profiles for two individual subclones of each transfectant in Fig. 4C, all of the mutant β4 subunits were expressed on the cell surface. To confirm that the mutant β4 subunits associated with endogenous α6 subunits, cell extracts of the β4 mutant-expressing subclones were immunoprecipitated with an α6-specific antibody and then immunoblotted with an antiserum that recognizes the C terminus of the β4 subunit. As shown in Fig. 4D, all of the mutant β4 subunits formed heterodimers with the endogenous α6 subunits. Moreover, all of the mutants were recognized by the C-terminal antiserum, which indicates that they are all expressed as full-length proteins.

To determine if the β4 cytoplasmic domain is phosphorylated on either Y1257 or Y1494 in response to α6β4 ligation, we assayed the tyrosine phosphorylation of the mutant β4 subunits after clustering the receptors with β4-specific antibodies. Mutation of either Y1257 or Y1494 resulted in a significant decrease in the level of β4-tyrosine phosphorylation (2 to 7% of the wild-type level) after clustering with β4-specific antibodies (Fig. 5). Addition of sodium orthovanadate to the cells during the clustering markedly increased the tyrosine phosphorylation of the mutant β4 subunits. However, when the results were normalized for total β4 protein, a significant decrease in the tyrosine phosphorylation of the mutant proteins compared to the level of phosphorylation of the wild-type β4 subunit was still observed.

Tyrosine 1494 in the β4 subunit is required for α6β4-dependent activation of PI3K. To evaluate the impact of mutating Y1257 and Y1494 in the β4-subunit cytoplasmic domain on the ability of the α6β4 receptor to activate downstream signaling pathways, we initially examined the ability of these α6β4 mutant receptors to promote increases in total cellular tyrosine

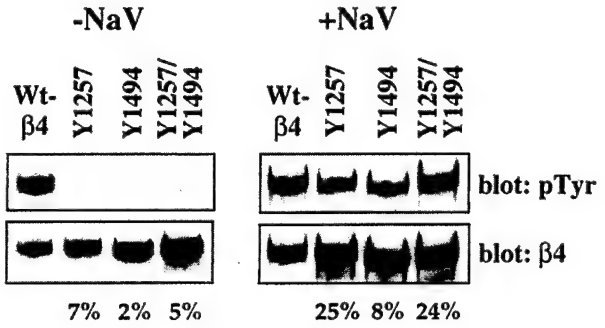

FIG. 5. Analysis of tyrosine phosphorylation of the β4 subunit. MDA-MB-435/β4 transfectants were incubated with β4-specific antibodies and allowed to adhere to anti-mouse IgG-coated plates for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with protein A-Sepharose for 2 h. The immune complexes were resolved by SDS–6% PAGE and then immunoblotted with RC-20. The immunoblots were subsequently stripped and reprobed with a β4-specific polyclonal antiserum. Shown are representative subclones expressing the wild-type (WT) β4 subunit and each of the β4 mutant subunits. The percentages of phosphorylation of the mutant β4 subunits compared to that of the wild-type β4 subunit are indicated below. NaV, sodium orthovanadate.

phosphorylation after clustering with β4-specific antibodies. MDA-MB-435 cells that expressed the wild-type β4 and each of the mutant β4 subunits were clustered with β4-specific antibodies, and the cell extracts were incubated with phosphotyrosine-specific antibody 4G10. As shown in Fig. 6A (top), ligation of wild-type α6β4 resulted in a marked increase in total cellular tyrosine phosphorylation levels. A similar increase in tyrosine phosphorylation was observed in two individual subclones that expressed the Y1257F β4 subunit, indicating that Y1257 is not essential for α6β4-dependent promotion of tyrosine phosphorylation (Fig. 6A). Although one of the Y1257F subclones had a lower level of tyrosine phosphorylation than was observed for the wild-type β4 subclone, this level of phosphorylation correlated with the levels of surface expression of the β4 subunit in these cells (Fig. 4C). In contrast, none of the MDA-MB-435 subclones that expressed the Y1494F or the double-mutant Y1257F/Y1494F β4 subunits showed increases in cellular tyrosine phosphorylation levels in response to α6β4 clustering. These results suggest that Y1494 is essential for α6β4-dependent promotion of tyrosine phosphorylation.

To determine if either Y1257 or Y1494 in the β4 cytoplasmic domain is required for PI3K activation by the α6β4 receptor, we assessed the association of PI3K with the phosphotyrosine immune complexes after ligation of α6β4 in the subclones that expressed these mutant β4 subunits. As shown in Fig. 6A (bottom), p85 association with the phosphotyrosine immune complexes increased after the clustering of the wild-type α6β4 and the Y1257F mutant α6β4 receptors. In contrast, an increase in p85 association with phosphotyrosine immune complexes in response to the clustering of the Y1494F and Y1257F/Y1494F mutant α6β4 receptors was not observed. The level of p85 subunit association with the phosphotyrosine immune complexes correlated well with the level of PI3K activity observed in in vitro kinase assays (Fig. 6B).

Our data suggest that IRS-2 is an important intermediate in the activation of PI3K by the α6β4 integrin. Therefore, we

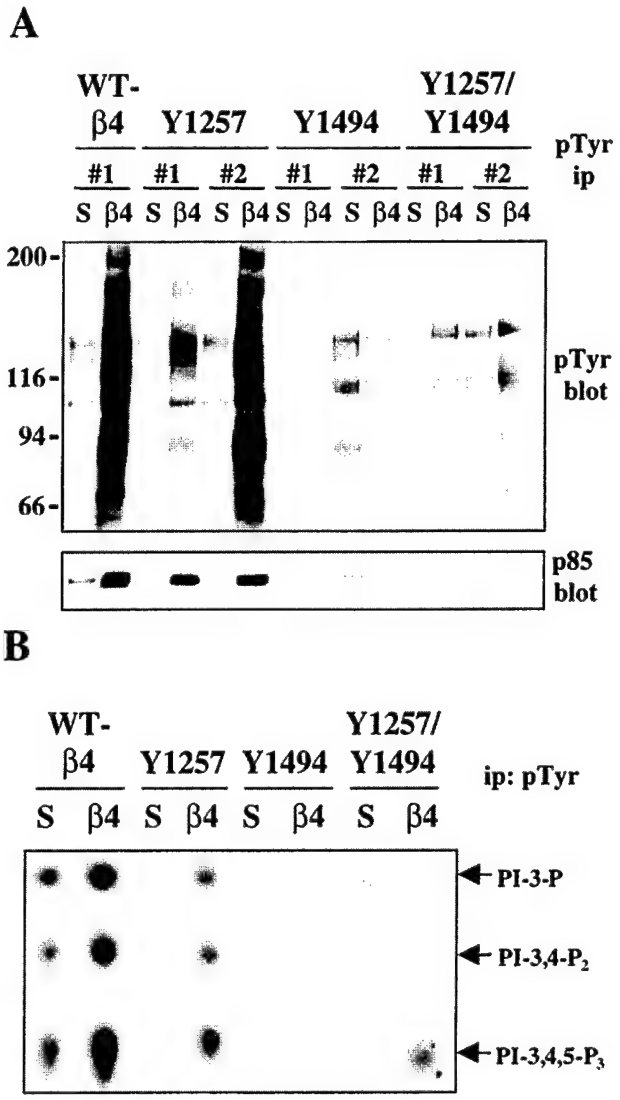

FIG. 6. Analysis of PI3K activity in the MDA-MB-435 mutant β4 transfectants. MDA-MB-435 transfectants were maintained in suspension or incubated with a β4-specific antibody (UM-A9) and allowed to adhere to anti-mouse IgG-coated plates for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with 4G10 and protein A-Sepharose for 3 h. (A) After being washed, the immune complexes were resolved by SDS–6% PAGE and immunoblotted with RC-20. Shown are one representative subclone expressing the wild-type (WT) β4 subunit and two representative subclones of each of the mutant β4 transfectants. (B) After being washed, the immune complexes were resuspended in a kinase reaction mixture and incubated for 10 min at room temperature. The phosphorylated lipids were resolved by thin-layer chromatography. Arrows, D3-phosphoinositides. S, cells maintained in suspension; β4, MDA-MB-435 cells clustered with the β4-specific antibody. ip, immunoprecipitation.

examined the ability of the mutant α6β4 receptors to promote IRS-2 tyrosine phosphorylation. As shown in Fig. 7A (top), mutation of Y1494 inhibited the ability of the α6β4 receptor to promote IRS-2 phosphorylation. Tyrosine phosphorylation of IRS-2 was also inhibited by the double Y1257F Y1494F β4 mutations. Moreover, the recruitment of p85 to IRS-2 in response to α6β4 ligation was prevented in the Y1494F-express-

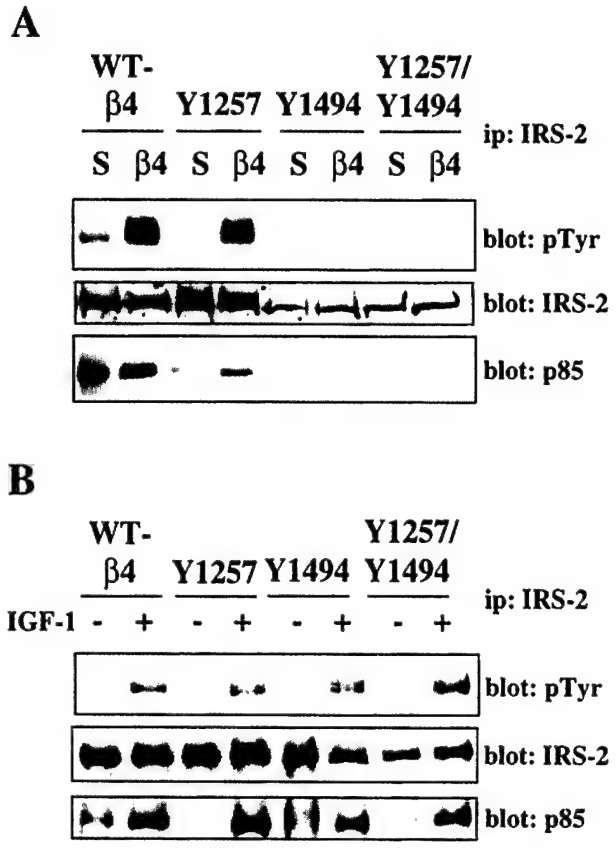

FIG. 7. Analysis of IRS-2 phosphorylation in the MDA-MB-435 mutant β4 transfectants. (A) MDA-MB-435 transfectants were maintained in suspension or incubated with β4-specific antibodies and allowed to adhere to anti-mouse IgG-coated plates for 30 min. WT, wild type; ip, immunoprecipitation. (B) MDA-MB-435 transfectants were incubated in the presence or absence of IGF-1 (100 ng/ml) for 5 min. Aliquots of cell extracts from equivalent numbers of cells were incubated with an IRS-2-specific antibody and protein A-Sepharose for 3 h. The immune complexes were resolved by SDS–6% PAGE and then immunoblotted with RC-20 (top). The immunoblots were subsequently stripped and reprobed with IRS-2-specified (middle) and p85-specific (bottom) polyclonal antisera.

ing and Y1257F Y1494F-expressing subclones (Fig. 6A, bottom). Although lower levels of IRS-2 expression were observed in the Y1494F-expressing and Y1257F- and Y1494F-expressing subclones, IRS-2 phosphorylation in response to α6β4 ligation was not detected even after prolonged exposure of the immunoblot. To confirm that the lack of IRS-2 phosphorylation in the subclones expressing the mutant β4 subunits was specific to α6β4-dependent signaling, the transfectants were treated with IGF-1, which promotes IRS-2 phosphorylation through IGF-1R. As shown in Fig. 7B, IRS-2 phosphorylation and PI3K recruitment in the wild-type and β4 mutant-expressing subclones after IGF-1 stimulation were equivalent. Taken together, our results indicate that Y1494 in the β4 subunit plays a pivotal role in the ability of the α6β4 integrin to activate PI3K.

Tyrosine 1494 in the β4 subunit is required for α6β4-dependent invasion. Expression of the β4 subunit increases the invasive potential of MDA-MB-435 cells, and we have hypothesized that this ability to promote invasion involves activation of PI3K by the α6β4 receptor (64). If this prediction is correct, expression of the Y1494F and Y1257F/Y1494F β4 mutant subunits in MDA-MB-435 cells should not increase their invasive potential. To address this question, subclones expressing the wild-type and mutant β4 subunits were assayed for their ability to invade Matrigel using a modified Boyden chamber assay. The MDA-MB-435 subclones expressing the Y1257F mutant subunit invaded to the same extent as the MDA-MB-435 subclone that expressed the wild-type β4 subunit (Fig. 8A). In contrast, the MDA-MB-435 subclones that expressed the Y1494F and Y1257F/Y1494F mutant β4 subunits did not invade. In fact, invasion was reduced below the level observed for the MDA-MB-435/mock transfectants, which suggests that the Y1494F and Y1257F/Y1494F mutant β4 subunits act in a dominant-negative manner for invasion.

The lack of invasion in the mutant β4 transfectants could result from a deficiency in adhesion. To examine if the observed decrease in invasion of the Y1494F and Y1257F/Y1494F transfectants was related to a decrease in cell adhesion, the ability of these cells to adhere to a laminin-1 or collagen I substrate was assessed. As shown in Fig. 8B, the Y1494F and Y1257F/Y1494F transfectants demonstrated a 1.5- to 2-fold higher level of adhesion to both substrates than the mock, wild-type β4, and Y1257 transfectants. The increased adhesion was not due to higher levels of receptor expression in the Y1494F and Y1257F/Y1494F transfectants (data not shown). Interestingly, the haptotactic migration of the Y1494F and Y1257F/Y1494F transfectants was significantly diminished on both laminin-1 and collagen I substrata (data not shown). The increased adhesive strength of the mutant β4 transfectants may inhibit their motility, which would impede the ability of these cells to invade (37). These results suggest that the Y1494 of the β4 subunit is important for regulating dynamic adhesion and that, in the absence of the IRS-2–PI3K signaling pathway activated through this tyrosine residue, other signals from the α6β4 receptor may promote stable adhesion.

DISCUSSION

Our results establish that the α6β4 integrin activates PI3K through the signaling adapters IRS-1 and IRS-2. This is a unique mechanism for α6β4 because the IRS proteins are not involved in PI3K activation downstream of the α6β1 receptor. We have also identified a specific tyrosine residue in the β4 cytoplasmic domain, Y1494, which is essential for the activation of PI3K and for the ability of the α6β4 integrin to promote carcinoma invasion. Taken together, our findings highlight a novel mechanism for α6β4-dependent signaling and for the ability of this integrin to promote carcinoma invasion.

Invasion is thought to be the essential first step for tumor cells in the metastatic cascade (14). The correlation between metastasis and patient mortality provides a strong impetus to elucidate the mechanisms involved in this transition. One approach to understanding how carcinoma cells acquire a motile, invasive phenotype is to dissect the cellular alterations that occur to drive this complex process. In this regard, previous work, including our own, has established that the α6β4 integrin can promote carcinoma invasion (12, 21, 64, 67). We determined that activation of PI3K was not only essential but also

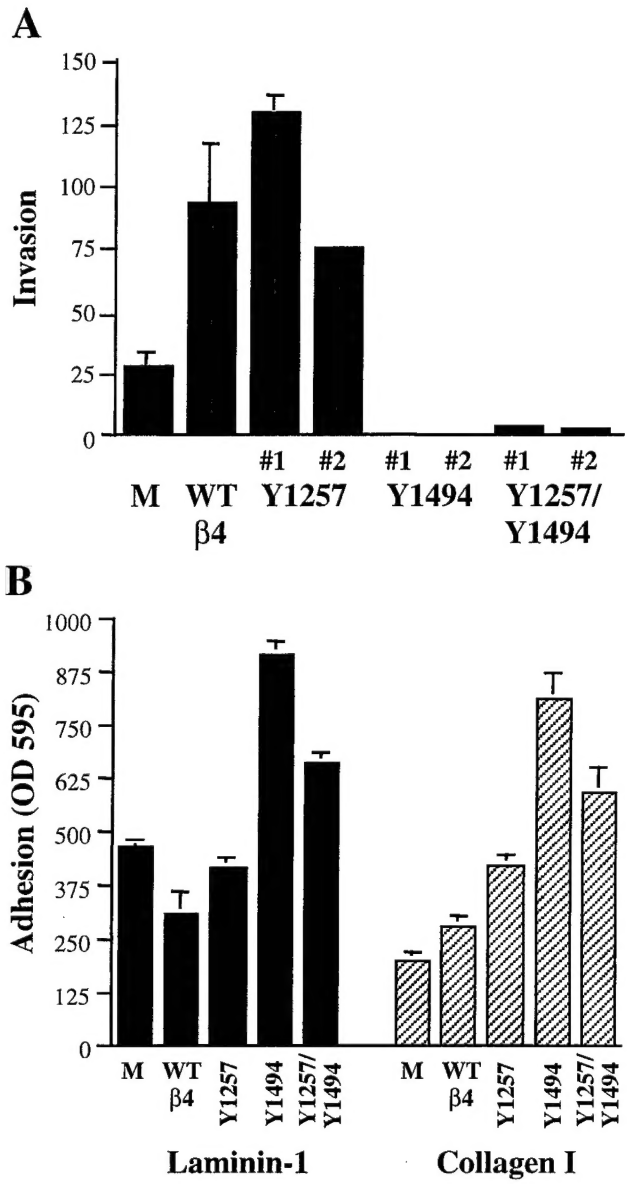

FIG. 8. Analysis of invasion and adhesion by the MDA-MB-435 mutant β4 transfectants. (A) MDA-MB-435 transfectants were assayed for their ability to invade Matrigel. Matrigel was diluted in cold distilled water, added to the upper well of Transwell chambers, and dried in a sterile hood. The Matrigel was reconstituted with medium, and the transfectants (5×10^4) were added to each well. Conditioned NIH 3T3 medium was added to the bottom wells of the chambers. After 4 h at 37°C, the cells that had not invaded were removed and the cells that had invaded to the lower surface of the filters were fixed, stained, and quantitated as described in Materials and Methods. The data shown are from one (mock and wild-type [WT] β4) or two (β4 mutants) individual subclones of each transfectant and are the mean values (± standard deviations [SD]) of a representative experiment done in triplicate. M, MDA-MB-435 cells transfected with vector alone; WT β4, MDA-MB-435 cells transfected with the wild-type β4 subunit. (B) MDA-MB-435 transfectants were assayed for their ability to adhere to laminin-1 and collagen I substrata. Forty-eight-well plates were coated overnight with 20 μg of laminin-1 or collagen I/ml (200 μl/well). The transfectants (10^5) were added to each well, and the plates were incubated for 1 h at 37°C. After being washed, the cells were fixed, stained, and quantitated as described in Materials and Methods. The data shown are the mean values (± SD) from a representative experiment done in triplicate.

sufficient to increase the invasive potential of carcinoma cells, which emphasized the importance of investigating further the mechanism of α6β4 activation of this signaling pathway (64). We have now added to our understanding of this "invasion pathway" by establishing that IRS proteins IRS-1 and IRS-2 are upstream mediators in the activation of PI3K by the α6β4 integrin. This is the first report of an involvement of the IRS family in tumor invasion, and it establishes a new area of research for these proteins, which have been studied primarily for their role in metabolic regulation (84).

The role of the IRS proteins in α6β4-dependent promotion of tumor progression adds to other studies that have demonstrated an involvement of the IRS family in cancer. IRS-1 and IRS-2 are essential downstream effectors of IGF-1R, which is frequently overexpressed in tumors and which is a prognostic indicator of tumor recurrence and reduced patient survival (39, 77). Although only a limited number of studies have been performed to address directly the contribution of IRS function to cancer, the data to date support an important role for these proteins in tumor biology. For example, in breast cancer, an essential role for IRS-1 in IGF-1-dependent breast carcinoma cell survival and an involvement in IGF-1-dependent breast carcinoma cell growth have been observed (50). IRS-1 expression is regulated by estrogen, and the levels of IRS-1 are decreased in response to antiestrogens such as tamoxifen and ICI 182,780 (25, 60). This regulation of IRS-1 expression has been hypothesized to be a mechanism by which these antiestrogens inhibit breast carcinoma growth. Finally, high levels of IRS-1 expression in primary human breast cancers predict a greater incidence of recurrence and a decreased patient survival rate (58). Increased IRS-1 expression levels have also been observed in early stages of hepatocellular carcinoma, and a dominant-negative IRS-1 protein can reverse the malignant phenotype of transformed hepatocellular carcinoma cell lines (49, 73). Finally, IRS-1 and IRS-2 are both overexpressed in pancreatic cancer (36). It is intriguing to speculate that the correlations of IRS expression with tumor progression are related at least in part to the functions of the IRS proteins downstream of the α6β4 integrin.

The identification of IRS-1 and IRS-2 as signaling intermediates for the α6β4 integrin is significant because these proteins have the potential to regulate multiple signaling pathways downstream of this integrin receptor. IRS-1 and IRS-2 are cytoplasmic adapter proteins that do not contain intrinsic kinase activity but rather function by recruiting proteins to surface receptors, where they organize signaling complexes (84). These proteins belong to the IRS family, which includes IRS-1, IRS-2, IRS-3, and IRS-4. IRS-1 and IRS-2 are expressed ubiquitously, whereas the IRS-3 and IRS-4 homologs are more restricted in their localization (84). All of the IRS family members have homology and contain multiple binding motifs that are essential for their interaction with downstream effectors, which can include PI3K, Grb-2, SHP-2, Nck, Fyn, phospholipase C-γ, and Crk (4, 40, 46, 47, 66, 68, 69). With their ability to recruit such a variety of signaling molecules, the IRS proteins, not surprisingly, have been implicated in numerous cellular functions including mitogenesis, cell survival, gene transcription, and glucose transport (84). With regard to the involvement of the IRS proteins in α6β4-dependent signaling, we have identified PI3K as one downstream effector that is

recruited to these adapter proteins in response to receptor ligation. The IRS-dependent activation of PI3K is important for the ability of α6β4 to promote invasion, an essential function of metastatic cells. Moreover, given the potential of the IRS proteins to interact with many other signaling effectors, other α6β4-dependent signals may also be regulated through these adapter proteins. For example, although we have observed MAPK activation in response to α6β4 ligation in the MDA-MB-435/β4 transfectants, we have not observed Shc phosphorylation, a proposed mechanism for MAPK activation (16, 43). An alternative mechanism for MAPK activation downstream of the α6β4 integrin could be IRS recruitment of Grb2. In support of this, we have not observed MAPK activation in the Y1494 mutant transfectants (data not shown).

Our demonstration that the α6β4 integrin is capable of stimulating the phosphorylation of both IRS-1 and IRS-2 raises the question of whether these homologs serve identical or distinct functions downstream of this integrin receptor. Although IRS-1 and IRS-2 share overall structural features and have some common effector binding sites, they also have unique phosphorylation sites (84). Furthermore, there are a number of reports that suggest that these homologs have different functions. For example, overexpression of IRS-1, but not IRS-2, in IRS-1 null fibroblasts restores IGF-1 stimulated cell cycle progression to the level observed in normal fibroblasts (8). In addition, IGF-1 stimulation of fetal brown adipocytes results in the association of Grb-2 with IRS-1 but not with IRS-2 (80). Differences in intracellular localization have also been demonstrated for IRS-1 and IRS-2, and this may explain some of the functional distinctions between these two homologs (28). The most striking evidence for functional differences in IRS-1 and IRS-2 comes from the phenotype of the IRS-1 and IRS-2 knockout mice. IRS-1 null mice are stunted in their growth, but they do not develop diabetes (1). In contrast, IRS-2 null mice develop insulin resistance in the liver and skeletal muscle and progressively lose their ability to regulate glucose homeostasis (85). The question of distinct IRS homolog function in carcinoma cells is relevant because differences in the expression and activity of IRS-1 and IRS-2 in tumor cells have been reported. For example, IRS-1 and IRS-2 are predominantly expressed in estrogen receptor positive (ER^+) and ER^- breast carcinoma cells, respectively (29). If IRS-1 and IRS-2 activate distinct downstream pathways, the function of the α6β4 receptor would depend on which IRS homolog was activated in response to receptor ligation. Therefore, although the α6β4 integrin can promote the phosphorylation of both IRS-1 and IRS-2, it is intriguing to speculate that α6β4-dependent signaling in carcinoma cells could be influenced by factors that differentially regulate the expression of the IRS proteins.

One issue that arises from our identification of IRS-1 and IRS-2 as downstream effectors of the α6β4 receptor is how the phosphorylation of these adapters is regulated by this integrin receptor. The IRS proteins were first discovered as signaling intermediates of the insulin receptor (IR), and they bind to a consensus sequence in the IR, where they are phosphorylated directly by the intrinsic receptor kinase domain (84). Although the IRS consensus binding site is also present in the IGF-1R and interleukin-4 (IL-4) receptor, not all receptors that promote IRS phosphorylation contain this binding motif, including the α6β4 integrin (15, 26, 32). In addition, none of these receptors possess intrinsic kinase domains. Therefore, alternative mechanisms for the recruitment and phosphorylation of the IRS proteins are necessary. Several models for α6β4-dependent activation of the IRS proteins could be proposed based on the mechanisms utilized by other members of this group of receptors that lack the IRS binding motif. One potential model involves the JAK family of tyrosine kinases, which includes JAK1, JAK2, JAK3, and Tyk2 (42). Several receptors, including those for alpha interferon, prolactin, growth hormone, and cytokines IL-2, -4, -7, and -15, associate with members of the JAK family, and these kinases recruit and phosphorylate the IRS proteins upon receptor stimulation (30, 53, 86). A second model involves the phosphorylation of the IRS proteins by members of the src-kinase family (17). However, it is not clear how the IRS proteins are recruited to receptor complexes for this src-dependent phosphorylation to occur. Focal adhesion kinase (FAK) has also been shown to associate with and promote phosphorylation of IRS-1 (38). However, the FAK-dependent phosphorylation may be indirect due to recruitment of src to the protein complexes. Finally, the possibility that the α6β4 receptor interacts with the IRS proteins through an association with other surface receptors is also valid. For example, the αvβ3 integrin can recruit IRS-1 indirectly to receptor complexes through an interaction with the insulin receptor (61, 83). The elucidation of the mechanism involved in the α6β4-dependent phosphorylation of the IRS proteins will provide additional targets for the disruption of tumor invasion.

We have identified a single amino acid in the β4 cytoplasmic domain, Y1494, which is involved in promoting the α6β4-IRS-PI3K invasion pathway. Mutation of this tyrosine residue inhibited not only α6β4-dependent activation of PI3K but also the ability of this receptor to promote carcinoma invasion. The fact that mutation of Y1494 had such a dramatic impact on α6β4-dependent signaling emphasizes the likelihood that this residue is an essential binding site for downstream effectors of the α6β4 receptor. We specifically selected Y1257 and Y1494 for mutation based on the location of these tyrosines within ITIM consensus binding motifs (79). The presence of both of these tyrosines within ITIM motifs might suggest that these residues have similar functions. However, it is clear that mutation of Y1257 in the β4 subunit does not disrupt the signaling functions of the α6β4 integrin, as was observed when Y1494 was mutated, even though both of these tyrosines appear to be phosphorylated in response to α6β4 ligation. These findings indicate that the two ITIM motifs in the β4 subunit do not function equally and that the specific sequences surrounding Y1257 and Y1494 must be important for regulating α6β4-dependent signals. ITIM motifs are known to be potential binding sites for the SH2 domains of tyrosine phosphatases SHP-1 and SHP-2 and lipid phosphatases SHIP-1 and SHIP-2 (79). SHP-1 and -2 phosphatases regulate tyrosine kinase signaling pathways, whereas the SHIP-1 and -2 phosphatases regulate the PI3K signaling pathway (reviewed in references 59 and 65). Disruption of either pathway could produce the phenotype we observed with the mutant receptors. For example, the phosphorylation of the β4 subunit is tightly regulated by tyrosine phosphatases, and this supports the potential involvement of SHP-2 (Fig. 5). The fact that IRS-2 is not phosphor-

ylated in response to ligation of the Y1494 mutant receptor indicates that this tyrosine could be involved in the recruitment of IRS-2 to the receptor. However, it is also possible that Y1494 is essential for recruiting the kinase that regulates the phosphorylation of IRS-2 or other essential binding sites in the β4 subunit.

In summary, we have identified a novel mechanism for the activation of PI3K by the α6β4 integrin that involves IRS-1 and IRS-2 and requires Y1494 in the β4 cytoplasmic domain. Activation of this α6β4-IRS-PI3K signaling pathway promotes carcinoma invasion.

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